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(54) Title: TUMOUR NECROSIS FACTOR BINDING LIGANDS (57) Abstract <p>The present invention relates to ligands which bind to human tumour necrosis factor alpha (TNF) in a manner such that upon binding of these ligands to TNF the biological activity of TNF is modified. In preferred forms the ligand binds to TNF in a manner such that the induction of endothelial procoagulant activity of the TNF is inhibited; the binding of TNF to receptors on endothelial cells is inhibited; the induction of fibrin deposition in the tumour and tumour regression activities of the TNF are enhanced; and the cytotoxicity and receptor binding activities of the TNF are unaffected or enhanced on tumour cells. The ligand is preferably an antibody, F(ab) fragment, single domain antibody (dABs) single chain antibody or a serum binding protein. It is preferred, however, that the ligand is a monoclonal antibody or F(ab) fragment thereof.</p>		

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TUMOUR NECROSIS FACTOR BINDING LIGANDSField of the Invention

The present invention relates to ligands which bind to human tumour necrosis factor alpha (TNF) in a manner such that upon binding the biological activity of TNF is modified. The type of modification shown here is distinct from previous descriptions of antibodies which bind to TNF alpha and inhibit all TNF alpha activity. The new discovery shows how the different activities of TNF alpha can be selectively inhibited or enhanced. In addition, the present invention relates to a composition comprising a molecule bound to TNF and to methods of therapy utilising TNF and molecules active against TNF.

Background of the Invention

Tumor necrosis factor alpha (TNF) is a product of activated macrophages first observed in the serum of experimental animals presensitized with Bacillus Calmette-Guerin or Corynebacterium parvum and challenged with endotoxin (LPS). Following the systematic administration of TNF haemorrhagic necrosis was observed in some transplantable tumours of mice while in vitro TNF caused cytolytic or cytostatic effects on tumour cell lines.

In addition to its host-protective effect, TNF has been implicated as the causative agent of pathological changes in septicemia, cachexia and cerebral malaria. Passive immunization of mice with a polyclonal rabbit serum against TNF has been shown to protect mice against the lethal effects of LPS endotoxin, the initiating agent of toxic shock, when administered prior to infection.

The gene encoding TNF has been cloned allowing the usefulness of this monokine as a potential cancer therapy agent to be assessed. While TNF infusion into cancer patients in stage 1 clinical trials has resulted in tumour regression, side-effects such as thrombocytopaenia,

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lymphocytopaenia, hepatotoxicity, renal impairment and hypertension have also been reported. These quite significant side-effects associated with the clinical use of TNF are predictable in view of the many known effects of TNF, some of which are listed in Table 1.

TABLE 1BIOLOGICAL ACTIVITIES OF TNF

-ANTI-TUMOUR
-ANTI-VIRAL
10 -ANTI-PARASITE

FUNCTION

cytotoxic action on tumour cells
pyrogenic activity
15 angiogenic activity
inhibition of lipoprotein lipase
activation of neutrophils
osteoclast activation
induction of endothelial, monocyte and tumour cell
20 procoagulant activity
induction of surface antigens on endothelial cells
induction of IL-6
induction of c-myc and c-fos
induction of EGF receptor
25 induction of IL-1
induction of TNF synthesis
induction of GM-CSF synthesis
increased prostaglandin and collagenase synthesis
induction of acute phase protein C3

30

Of particular importance is the activation of coagulation which occurs as a consequence of TNF activation of endothelium and also peripheral blood monocytes. Disseminated intravascular coagulation is
35 associated with toxic shock and many cancers including

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gastro-intestinal cancer, cancer of the pancreas, prostate, lung, breast and ovary, melanoma, acute leukaemia, myeloma, myeloproliferative syndrome and myeloblastic leukaemia. Clearly modifications of TNF activity such that tumour regression activity remains intact but other undesirable effects such as activation of coagulation are removed or masked would lead to a more advantageous cancer therapy, while complete abrogation of TNF activity is sought for successful treatment of toxic shock.

Segregation of hormonal activity through the use of site-specific antibodies (both polyclonal and monoclonal) can result in enhanced hormonal activity (Aston et al, 1989, Mol. Immunol. 26, 435). To date few attempts have been made to assign antigenicity or function to particular regions of the TNF molecule for which the three-dimensional structure is now known. Assignment of function to such regions would permit the development of MAb and other ligands of therapeutic use. Polyclonal antibodies to amino acids 1 to 15 have been reported to block Hela R19 cell receptor binding by TNF (Socher et al, 1987, PNAS 84, 8829) whilst monoclonal antibodies recognising undefined conformational epitopes on TNF have been shown to inhibit TNF cytotoxicity in vitro (Bringman and Aggarwal, 1987, Hybridoma 6, 489). However, the effects of these antibodies on other TNF activities is unknown.

Description of the Present Invention

The present inventors have produced panels of monoclonal antibodies active against human TNF and have characterised them with respect to their effects on the anti-tumour effect of TNF (both in vitro and in vivo), TNF receptor binding, activation of coagulation (both in vitro and in vivo) and defined their topographic specificities. This approach has led the inventors to show that different topographic regions of TNF alpha are associated with

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different activities. Therefore the inventors enable the identification of antibodies or ligands which selectively enhance or inhibit TNF alpha activity, thereby providing for improved therapeutic agents and regimes including TNF
5 alpha.

In a first aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the following biological activities of the TNF are inhibited:-

- 10 1. Tumour regression;
2. Induction of endothelial procoagulant;
3. Induction of tumour fibrin deposition;
4. Cytotoxicity; and
5. Receptor binding.

15 In a preferred embodiment of all aspects the present invention the ligand is selected from the group consisting of antibodies, F(ab) fragments, restructured antibodies (CDR grafted humanised antibodies) single domain antibodies (dAbs), single chain antibodies, serum binding
20 proteins, receptors and natural inhibitors. The ligand may also be a protein or peptide which has been synthesised and which is analogous to one of the foregoing fragments. However, it is presently preferred that the ligand is a monoclonal antibody or F(ab) fragment thereof.

25 In a second aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant, tumour regression, induction of tumour fibrin deposition, cytotoxicity and receptor
30 binding activities of the TNF are inhibited, the ligand binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 1-18, 58-65, 115-125 and 138-149, or the topographic region of residues 1-18, 108-128, or the topographic region of
35 residues 56-79, 110-127 and 135-155 is substantially

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prevented from binding to naturally occurring biologically active ligands.

In a third aspect the present invention consists in a ligand which binds to human TNF in at least two regions
5 selected from the group consisting predominantly of the topographic region of residues 1-20, the topographic region of residues 56-77, the topographic region of residues 108-127 and the topographic region of residues 138-149.

10 In a preferred embodiment of the third aspect of the present invention the ligand binds to human TNF in the topographic regions of residues 1-18, 58-65, 115-125 and 138-149. Such sequence regions are topographically represented in Fig. 23.

15 In a further preferred embodiment of the third aspect of the present invention the ligand binds to human TNF in the topographic regions of residues 1-18 and 108-128. Such sequence regions are topographically represented in Fig. 24.

20 In a further preferred embodiment of the second aspect of the present invention the ligand binds to human TNF in the topographic regions of residues 56-79, 110-127 and 136-155. Such sequence regions are topographically represented in Fig. 25.

25 In a particularly preferred embodiment of the first, second and third aspects of the present invention the ligand is a monoclonal antibody selected from the group consisting of the monoclonal antibodies designated MAb 1, MAb 47 and MAb 54. Samples of the hybridoma cell lines
30 which produce MAb 1, MAb 54 and MAb 47 have been deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury,
35 Wiltshire SP4 0JG, United Kingdom. MAb 1 was deposited on

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3 August 1989 and accorded accession No. 89080301; MAb 54 was deposited on 31 August 1989 and accorded accession No. 89083103; MAb 47 was deposited on 14 December 1989 and accorded accession No. 89121402.

5 In a fourth aspect the present invention consists in a composition comprising TNF in combination with the ligand of the first, second or third aspect of the present invention, characterised in that the ligand is bound to the TNF.

10 In a fifth aspect the present invention consists in a method of treating toxic shock comprising administering either the ligand of the first, second or third aspect of the present invention or the composition of the fourth aspect of the present invention.

15 In a sixth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited; binding of TNF to receptors on endothelial
20 cells is inhibited; the induction of tumour fibrin deposition and tumour regression activities of the TNF are enhanced; the cytotoxicity is unaffected and tumour receptor binding activities of the TNF are unaffected or enhanced.

25 In a seventh aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited; the binding of the TNF to receptors on
30 endothelial cells is inhibited, the induction of tumour fibrin deposition and tumour regression activities of the TNF are enhanced; and the cytotoxicity and receptor binding activities of the TNF are unaffected; the ligand binding to the TNF such that the epitope of the TNF
35 defined by the topographic regions of residues 1-30,

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117-128 and 141-153 is substantially prevented from binding to naturally occurring biologically active ligands.

In an eighth aspect the present invention consists of a ligand which binds to human TNF in the topographic regions of residues 1-30, 117-128 and 141-153.

In a preferred embodiment of the eighth aspect of the present invention the ligand binds to human TNF in the topographic regions of residues 1-26, 117-128 and 141-153. Such sequence regions are topographically represented in Fig. 26.

In a preferred embodiment of the sixth, seventh and eighth aspects of the present invention the ligand is the monoclonal antibody designated MAb 32. A sample of the hybridoma producing MAb 32 was deposited with The European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom on 3 August 1989 and was accorded accession No. 89080302.

In a ninth aspect the present invention consists in a composition comprising TNF in combination with a ligand of the sixth, seventh or eighth aspects of the present invention characterised in that the ligand is bound to TNF. No previous documentation of administering MABs with TNF in order to modify activity of the administered cytokine exists.

In a tenth aspect the present invention consists in a method of treating tumours the growth of which is inhibited by TNF, comprising administering either the ligand of the sixth, seventh or eighth aspects of the present invention or the composition of the ninth aspect of the present invention.

In an eleventh aspect the present invention consists in a ligand which binds to residues 1-18 of human TNF

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(peptide 301).

In a twelfth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the induction
5 of endothelial procoagulant activity of the TNF is inhibited; the binding of TNF to receptors on endothelial cells is inhibited; the induction of tumour fibrin deposition and tumour regression activities of the TNF are enhanced; the cytotoxicity of the TNF are unaffected and
10 tumour receptor binding activities of the TNF are unaffected or enhanced, the ligand binding to TNF such that the epitope of the TNF defined by the topographic region of residues 1-18 is substantially prevented from binding to naturally occurring biologically active ligands.

15 In a thirteenth aspect the present invention consists in a composition comprising TNF in combination with a ligand of the eleventh or twelfth aspects of the present invention characterized in that the ligand is bound to the TNF.

20 In a fourteenth aspect the present invention consists in a method of treating tumours the growth of which is inhibited by TNF, comprising administering either the ligand of the eleventh or twelfth aspect of the present invention or the composition of the thirteenth aspect of
25 the present invention.

In a fifteenth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the cytotoxicity and tumour regression activities of the TNF
30 are unaffected; the induction of endothelial procoagulant and induction of tumour fibrin deposition activities of the TNF are inhibited and receptor binding activities of the TNF are unaffected.

In a sixteenth aspect the present invention consists
35 in a ligand capable of binding to human TNF, the ligand

being characterized in that when it binds to TNF the cytotoxicity and tumour regression activities of the TNF are unaffected; the induction of endothelial procoagulant and induction of tumour fibrin deposition activities of the TNF are inhibited and the tumour receptor binding activities of the TNF are unaffected, the ligand binding to TNF such that the epitope of the TNF defined by the topographic regions of residues 22-40, 49-97, 110-127 and 136-153 is substantially prevented from binding to naturally occurring biologically active ligands.

In a seventeenth aspect the present invention consists in a ligand which binds to human TNF in the topographic regions of residues 22-40, 49-97, 110-127 and 136-153. Such sequence regions are topographically represented in Fig. 27.

In a preferred embodiment of the seventeenth aspect of the present invention the ligand binds to human TNF in the topographic regions of residues 22-40, 49-96, 110-127 and 136-153. These regions being proximate in the 3D structure of TNF alpha.

In a preferred embodiment of the fifteenth, sixteenth and seventeenth aspects of the present invention the ligand is the monoclonal antibody designated MAb 42. A sample of the hybridoma cell line producing MAb 42 was deposited with The European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom on 3 August 1989 and was accorded accession No. 89080304.

In an eighteenth aspect the present invention consists in a composition comprising TNF in combination with the ligand of the fifteenth, sixteenth or seventeenth aspects of the present invention, characterised in that the ligand is bound to the TNF.

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In a nineteenth aspect the present invention consists in a method of treating tumours inhibited by the action of TNF comprising administering the ligand of the fifteenth, sixteenth or seventeenth aspects of the present invention
5 or the composition of the eighteenth aspect of the present invention.

In a twentieth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the
10 tumour fibrin deposition activity of the TNF is enhanced; the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity, tumour regression and receptor binding activities of the TNF are inhibited.

In a twenty-first aspect the present invention
15 consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the tumour fibrin deposition activity of the TNF is enhanced; the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity,
20 tumour regression and tumour receptor binding activities of the TNF are inhibited, the ligand binding to TNF such that the epitope of the TNF defined by the topographic regions of residues 12-22, 36-45, 96-105 and 132-157 is substantially prevented from binding to naturally
25 occurring biologically active ligands.

In a twenty-second aspect the present invention consists in a ligand which binds to human TNF in the topographic regions of residues 12-22, 36-45, 96-105 and 132-157. These regions are proximate in the 3D structure
30 of TNF and are topographically represented in Fig. 28.

In a preferred embodiment of the twentieth, twenty-first and twenty-second aspects of the present invention the ligand is the monoclonal antibody designated MAb 25. A sample of the hybridoma cell line producing MAb
35 25 was deposited with the European Collection of Animal

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Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom on 14 December 1989 and

5 was accorded accession No. 89121401.

In a twenty-third aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the tumour fibrin deposition activity of the TNF is
10 enhanced and the cytotoxicity, tumour regression, induction of endothelial procoagulant and receptor binding activities of the TNF are inhibited.

In a twenty-fourth aspect the present invention consists in a ligand capable of binding to human TNF, the
15 ligand being characterized in that when it binds to TNF the tumour fibrin deposition activity of the TNF is enhanced and the cytotoxicity, tumour regression, induction of endothelial procoagulant and tumour receptor binding activities of the TNF are inhibited, the ligand
20 binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 1-20 and 76-90 is substantially prevented from binding to naturally occurring biologically active ligands.

In a twenty-fifth aspect the present invention
25 consists in a ligand which binds to human TNF in the topographic regions of residues 1-20 and 76-90. These regions are proximate in the 3D structure of TNF and are topographically represented in Fig. 29.

In a preferred embodiment of the twenty-fifth aspect
30 of the present invention the ligand binds to TNF in the topographic regions of residues 1-18 and 76-90.

In a preferred embodiment of the twenty-third, twenty-fourth and twenty-fifth aspects of the present invention the ligand is the monoclonal antibody designated
35 MAb 21. A sample of the hybridoma cell line producing MAB

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21 was deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom on 25 January 1990 and was accorded accession No. 90012432.

In a twenty-sixth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the fibrin deposition activity of the TNF is unaffected and the cytotoxicity, tumour regression, induction of endothelial procoagulant and tumour receptor binding activities of the TNF are inhibited.

In a twenty-seventh aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the tumour fibrin deposition activity of the TNF is unaffected and the cytotoxicity, tumour regression, induction of endothelial procoagulant and receptor binding activities of the TNF are inhibited, the ligand binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 22-40, 69-97, 105-128 and 135-155 is substantially prevented from binding to naturally occurring biologically active ligands.

In an twenty-eighth aspect the present invention consists in a ligand which binds to human TNF in the topographic regions of residues 22-40, 69-97, 105-128 and 135-155. These regions are proximate in the 3D structure of TNF and are topographically represented in Fig. 30.

In a preferred embodiment of the twenty-sixth, twenty-seventh and twenty-eighth aspects of the present invention the ligand is the monoclonal antibody designated MAb 53. A sample of the hybridoma cell line producing MAb 53 was deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production

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Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG, United Kingdom on 25 January 1990 and was accorded accession No. 90012433.

5 In a twenty-ninth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to the TNF tumour fibrin deposition, induction of endothelial procoagulant, cytotoxicity, tumour regression and receptor
10 binding activities of the TNF are unaffected.

In a thirtieth aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the tumour fibrin deposition, induction of endothelial
15 procoagulant, cytotoxicity, tumour regression and receptor binding activities of the TNF are unaffected, the ligand binding to TNF such that the epitope of the TNF defined by the topographic regions of residues 22 - 31 and 146 - 157 is substantially prevented from binding to naturally
20 occurring biologically active ligands.

In a thirty-first aspect the present invention consists in a ligand which binds to human TNF in the topographic regions of residues 22-31 and 146-157. These regions are proximate in the 3D structure of TNF and are
25 topographically represented in Fig. 31.

In a preferred embodiment of the twenty-ninth, thirtieth and thirty-first aspects of the present invention the ligand is the monoclonal antibody designated MAb 37. A sample of the hybridoma cell line producing MAb
30 37 was deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG, United Kingdom on 3 August 1989 and was
35 accorded accession No. 89080303.

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In a thirty-second aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity, tumour regression, tumour fibrin deposition, and receptor binding activities of the TNF are inhibited.

In a thirty-third aspect the present invention consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity, tumour regression, tumour fibrin deposition and receptor binding activities of the TNF are inhibited, the ligand binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 22 - 40 and 49 - 98 is substantially prevented from binding to naturally occurring biologically active ligands.

In a thirty-fourth aspect the present invention consists in a ligand which binds to human TNF in at least one of the regions selected from the group consisting of the topographic region of residues 22-40, the topographic region of residues 49-98 and the topographic region of residues 69-97.

In a preferred embodiment of the thirty-fourth aspect of the present invention the ligand binds to human TNF in the topographical region of residues 49-98. This region is topographically represented in Fig. 32.

In a further preferred embodiment of the thirty-fourth aspect of the present invention the ligand binds to human TNF in the topographic regions of residues 22-40 and 70-87. These regions are proximate in the 3D structure of TNF and are topographically represented in Fig. 33.

In a preferred embodiment of the thirty-second,

thirty-third and thirty-fourth aspects of the present invention the ligand is monoclonal antibody MAb 11 or MAb 12.

In a thirty-fifth aspect the present invention
5 consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited.

In a thirty-sixth aspect the present invention
10 consists in a ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited, the ligand binding to TNF such that the epitope of the TNF defined by the topographical region of
15 residues 108 - 128 is prevented from binding to naturally occurring biologically active ligands.

In a thirty-seventh aspect the present invention consists in a ligand which binds to human TNF in the topographical region of residues 108 - 128.

20 In a preferred embodiment of the thirty-fifth, thirty-sixth and thirty-seventh aspects of the present invention the ligand is selected from the group consisting of monoclonal antibodies designated MAb 1, MAb 32, MAb 42, MAb 47, MAb 53 and MAb 54.

25 The biological activities of TNF referred to herein by the terms "Tumour Regression", "Induction of Endothelial Procoagulant", "Induction of Tumour Fibrin Deposition", "Cytotoxicity" and "Receptor Binding" are to be determined by the methods described below.

30 The term "single domain antibodies" as used herein is used to denote those antibody fragments such as described in Ward et al (Nature, Vol. 341, 1989, 544 - 546) as suggested by these authors.

In order that the nature of the present invention may
35 be more clearly understood, preferred forms thereof will

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now be described with reference to the following example and accompanying figures in which:-

Fig. 1 shows the results of a titration assay with MAb 1 against TNF;

5 Fig. 2 shows TNF MAb 1 scatchard plot and affinity determination;

Fig. 3 shows the effect of anti-TNF monoclonal antibodies 1 and 32 on TNF cytotoxicity in WEHI-164 cells;

10 Fig. 4 shows the effect of MAb 1 on TNF-induced regression of a Meth A solid tumour;

Fig. 5 shows the effect of MABs 1 and 25 on TNF-induced Meth A Ascites tumour regression;

Fig. 6 shows the effect of anti-TNF MABs on induction of endothelial cell procoagulant activity by TNF;

15 Fig. 7 shows incorporation of labelled fibrinogen into tumours of tumour-bearing mice and the effect of anti-TNF MABs;

Fig. 8 is a schematic representation of epitopes on TNF;

20 Fig. 9 shows the effect of anti-TNF MABs on TNF-induced regression of WEHI-164 tumours;

Fig. 10 shows the enhancement of TNF regression activity by MAB 32 in two experiments;

25 Fig. 11 shows the enhancement of TNF-induced tumour regression by MAB 32 - dose response at day 1 and day 2;

Fig. 12 shows binding of radio labelled TNF to receptors on bovine aortic endothelial cells;

30 Fig. 13 shows receptor binding studies of TNF complexed with MAB 32 (—◆—), control antibody (—□—) and MAB 47 (—■—) on melanoma cell line MM418E;

Fig. 14 shows receptor binding studies of TNF complexed with MAB 32 (—◆—), control antibody (—□—) and MAB 47 (—■—) on melanoma cell line IGR3;

35 Fig. 15 shows receptor binding studies of TNF complexed with MAB 32 (—◆—), control antibody (—□—)

and MAb 47 (—■—) on bladder carcinoma cell line 5637;

Fig. 16 shows receptor binding studies of TNF complexed with MAb 32 (—◆—), control antibody (—□—) and MAb 47 (—■—) on breast carcinoma cell line MCF7;

5 Fig. 17 shows receptor binding studies of TNF complexed with MAb 32 (—◆—), control antibody (—□—) and MAb 47 (—■—) on colon carcinoma cell line B10;

Fig. 18 shows the effect on TNF-mediated tumour regression in vivo by MAb 32 (■) control MAb (□)
10 and MAb 47 (*);

Fig. 19 shows the effect on TNF-mediated tumour regression in vivo by control MAb, MAb 32 and univalent FAb' fragments of MAb 32;

Fig. 20 shows the effect on TNF induced tumour
15 regression by control MAb (■), MAb 32 (■) and peptide 301 antiserum (■);

Fig. 21 shows MAb 32 reactivity with overlapping peptides of 10 AA length; and

Fig. 22 shows a schematic three dimensional
20 representation of the TNF molecule.

Fig. 23 shows topographically the region of residues 1 - 20, 56 - 77, 108 - 127 and 138 - 149;

Fig. 24 shows topographically the region of residues 1 - 18 and 108 - 128;

25 Fig. 25 shows topographically the region of residues 56 - 79, 110 - 127 and 136 - 155;

Fig. 26 shows topographically the region of residues 1 - 26, 117 - 128 and 141 - 153;

Fig. 27 shows topographically the region of residues
30 22 - 40, 49 - 97, 110 - 127 and 136 - 153;

Fig. 28 shows topographically the region of residues 12 - 22, 36 - 45, 96 - 105 and 132 - 157;

Fig. 29 shows topographically the region of residues 1 - 20 and 76 - 90;

35 Fig. 30 shows topographically the region of residues

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22 - 40, 69 - 97, 105 - 128 and 135 - 155;

Fig. 31 shows topographically the region of residues

22 - 31 and 146 - 157;

Fig. 32 shows topographically the region of residues

5 49 - 98; and

Fig. 33 shows topographically the region of residues

22-40 and 70-87.

Animals and Tumour Cell Lines

In all experiments BALB/C female mice aged 10-12
10 weeks obtained from the CSIRO animal facility were used.
Meth A solid tumour and Meth A ascites tumour cell lines
were obtained from the laboratory of Dr. Lloyd J. Old
(Sloan Kettering Cancer Centre) and the WEHI-164
fibrosarcoma line was obtained from Dr. Geeta Chaudhri
15 (John Curtin School of Medical Research, Australian
National University).

Fusions and Production of Hybridomas

Mice were immunised with 10 ug human recombinant TNF
intra-peritoneally in Freund's complete adjuvant. One
20 month later 10 ug TNF in Freund's incomplete adjuvant was
administered. Six weeks later and four days prior to
fusion selected mice were boosted with 10 ug TNF in PBS.
Spleen cells from immune mice were fused with the myeloma
Sp2/0 according to the procedure of Rathjen and Underwood
25 (1986, Mol. Immunol. 23, 441). Cell lines found to
secrete anti-TNF antibodies by radioimmunoassay were
subcloned by limiting dilution on a feeder layer of mouse
peritoneal macrophages. Antibody subclasses were
determined by ELISA (Misotest, Commonwealth Serum
30 Laboratories).

Radioimmunoassay

TNF was iodinated using lactoperoxidase according to
standard procedures. Culture supernatants from hybridomas
(50 ul) were incubated with ¹²⁵I TNF (20,000 cpm in 50 ul)
35 overnight at 4°C before the addition of 100 ul Sac-Cel

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(donkey anti-mouse/rat immunoglobulins coated cellulose, Wellcome Diagnostics) and incubated for a further 20 minutes at room temperature (20°C). Following this incubation 1 ml of PBS was added and the tubes centrifuged at 2,500 rpm for 5 minutes. The supernatant was decanted and the pellet counted for bound radioactivity.

Antibody-Antibody Competition Assays

The comparative specificities of the monoclonal antibodies were determined in competition assays using either immobilized antigen (LACT) or antibody (PACT) (Aston and Ivanyi, 1985, Pharmac. Therapeut. 27, 403).

PACT

Flexible microtitre trays were coated with monoclonal antibody (sodium sulphate precipitated globulins from mouse ascites fluid, 100 micrograms per ml in sodium bicarbonate buffer, 0.05M, pH 9.6) overnight at 4°C prior to blocking non-specific binding sites with 1% bovine serum albumin in PBS (BSA/PBS). The binding of ¹²⁵I TNF to immobilised antibody was determined in the presence of varying concentrations of a second anti-TNF monoclonal antibody. Antibody and TNF were added simultaneously and incubated for 24 hours prior to washing with PBS (4 times) and counting wells for bound radioactivity. 100% binding was determined in the absence of heterologous monoclonal antibody while 100% competition was determined in the presence of excess homologous monoclonal antibody. All dilutions were prepared in BSA/PBS.

LACT

The binding of protein A purified, radiolabelled monoclonal antibodies to TNF coated microtitre wells was determined in the presence of varying concentrations of a second monoclonal antibody. Microtitre plates were coated with TNF (50 micrograms per ml) as described above. Quantities of competing antibodies (50 microlitres) were

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pre-incubated on plates for 4 hour at 4°C prior to addition of 125I monoclonal antibody (30,000 cpm) for a further 24 hours. Binding of counts to wells was determined after four washes with PBS. 100% binding was determined in the absence of competing antibody while 100% competition was determined in the presence of excess unlabelled monoclonal antibody.

WEHI-164 Cytotoxicity Assay

Bioassay of recombinant TNF activity was performed according to Espevik and Nissen-Meyer (1986, J. Immunol. Methods 95, 99). The effect of the monoclonal antibody on TNF activity was determined by the addition of the monoclonal antibody to cell cultures at ABT90.

Tumour Regression Experiments

Modulation of TNF-induced tumour regression activity by monoclonal antibodies was assessed in three tumour models: the subcutaneous tumours WEHI-164 and Meth A sarcoma and the ascitic Meth A tumour. Subcutaneous tumours were induced by the injection of approximately 5×10^5 cells. This produced tumours of between 10 - 15 mm approximately 14 days later. Mice were injected intra-peritoneally with human recombinant TNF (10 micrograms) plus monoclonal antibody (200 microlitres ascites globulin) for four consecutive days. Control groups received injections of PBS alone or TNF plus monoclonal antibody against bovine growth hormone. At the commencement of each experiment tumour size was measured with calipers in the case of solid tumours or tumour-bearing animals weighed in the case of ascites mice. These measurements were taken daily throughout the course of the experiment.

Radio-Receptor Assays

WEHI-164 cells grown to confluency were scrape harvested and washed once with 1% BSA in Hank's balanced salt solution (HBSS, Gibco). 100 ul of unlabelled TNF

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(1-10,000 ng/tube) or monoclonal antibody (10 fold dilutions commencing 1 in 10 to 1 in 100,000 of ascitic globulin) was added to 50ul 125I TNF (50,000 cpm). WEHI cells were then added (200 microlitres containing 2 x 10⁶ cells). This mixture was incubated in a shaking water bath at 37°C for 3 hours. At the completion of this incubation 1 ml of HBSS was added and the cells spun at 16,000 rpm for 30 seconds. The supernatant was discarded and bound 125I TNF in the cell pellet counted.

10 All dilutions were prepared in HBSS containing 1% BSA.

Procoagulant Induction by TNF on Endothelial Cells

Bovine aortic endothelial cells (passage 10) were grown in RPMI-1640 containing 10% foetal calf serum (FCS), penicillin, streptomycin, and 2-mercaptoethanol at 37°C in 5% CO₂. For induction of procoagulant activity by TNF the cells were trypsinised and plated into 24-well Costar trays according to the protocol of Bevilacqua et al., 1986 (PNAS 83, 4533). TNF (0-500 units/culture) and monoclonal antibody (1 in 250 dilution of ascitic globulin) was added after washing of the confluent cell monolayer with HBSS. After 4 hours the cells were scrape harvested, frozen and sonicated. Total cellular procoagulant activity was determined by the recalcification time of normal donor platelet-poor plasma performed at 37°C, 100 microlitres of citrated platelet-poor plasma was added to 100 ul of cell lysate and 100 ul of calcium chloride (30mM) and the time taken for clot formation recorded. In some experiments tumour cell culture supernatant was added to endothelial cells treated with TNF and/or monoclonal antibody (final concentration of 1 in 2).

Incorporation of 125I Fibrinogen into Tumours of Mice Treated with TNF and Monoclonal Antibody

In order to examine the effect of TNF and monoclonal antibodies on fibrin formation in vivo, BALB/c mice were

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injected subcutaneously with WEHI-164 cells (10^5 cells/animal). After 7 - 14 days, when tumours reached a size of approximately 1 cm in diameter, animals were injected intra-peritoneally with TNF (10 ug/animal) and 125I human fibrinogen (7.5ug/animal, 122uCi/mg Amersham) either alone or in the presence of monoclonal antibody to human TNF (200ul/animal ascitic globulin). Monoclonal antibody against bovine growth hormone was used as control monoclonal antibody. Two hours after TNF infusion incorporation of 125I fibrinogen into mouse tissue was determined by removing a piece of tissue, weighing it and counting the sample in a gamma counter.

In all 13 monoclonal antibodies reacting with human TNF were isolated. These monoclonal antibodies were designated MAb 1, MAb 11, MAb 12, MAb 20, MAb 21, MAb 25, MAb 31, MAb 32, MAb 37, MAb 42, MAb 47, MAb 53 and MAb 54. The effect of these monoclonal antibodies on the bioactivity of human TNF is set out in Table 2.

As can be seen from Table 2, whilst some monoclonal antibodies inhibit both anti-tumour activity and activation of coagulation by human TNF (MAb 1, 47 and 54) not all antibodies which inhibit the anti-tumour activity inhibit activation of coagulation either in vitro or in vivo (MAb 11, 12, 25 and 53). Indeed MAb 21 which inhibited tumour regression enhanced the activation of coagulation in vivo.

TABLE 2
EFFECT OF MONOCLONAL ANTIBODIES ON TNF BIOACTIVITY

		<u>MONOCLONAL ANTIBODY</u>												
5	TNF BIOACTIVITY	1	11	12	20	21	25	31	32	37	42	47	53	54
	Cytotoxicity	-	-	-	0	-	-	0	0	0	0	-	-	-
10	Tumour Regression	-	-	-	0	-	-	0	+	0	0	-	-	-
	Induction of Procoagulant (Endothelial)	-	0	0	-	-	0	0	-	0	-	-	-	-
15	Fibrin Deposition (tumour)	-	-	-	+	+	+	+	+	0	-	-	0	-
20	Receptor Binding (WEHI-164)	-	-	-	0	-	-	0	+ / 0*	0	0	-	-	-

25 + Enhancement
0 No effect
- Inhibition
* Depending on MAb concentration in the case of WEHI-164 tumour cells and tumour type (see Figs. 3, 13 - 17).

30 MABs 1, 47 and 54, which have been shown in
competition binding studies to share an epitope on TNF,
can be seen to have highly desirable characteristics in
treatment of toxic shock and other conditions of
bacterial, viral and parasitic infection where TNF levels
35 are high requiring complete neutralisation of TNF. Other
monoclonal antibodies such as MAB 32 are more appropriate
as agents for coadministration with TNF during cancer
therapy since they do not inhibit tumour regression but do
inhibit activation of coagulation. This form of therapy
40 is particularly indicated in conjunction with cytotoxic
drugs used in cancer therapy which may potentiate
activation of coagulation by TNF (e.g. vinblastin,

acyclovir, IFN alpha, IL-2, actinomycin D, AZT, radiotherapy, adriamycin, mytomycin C, cytosine arabinoside, dounorubicin, cis-platin, vincristine, 5-flurouracil, bleomycin, (Watanabe N et al 1988

5 Immunopharmacol. Immunotoxicol. 10 117-127) or in diseases where at certain stages TNF levels are low (e.g. AIDS) and where individuals may have AIDS associated cancer e.g. Kaposi sarcoma, non-Hodgkins lymphoma and squamous cell carcinoma.

10 Monoclonal antibody MAb 1 has been found to have the following characteristics:-

1. Binds human recombinant TNF alpha, but not human lymphotoxin (TNF beta) or human interferon. Similarly MAb 1 does not cross-react with recombinant
15 murine TNF (Fig.1).
2. MAb 1 is of the immunoglobulin type IgG1, K with an apparent affinity of 4.4×10^{-9} moles/litre (Fig. 2).
3. MAb neutralises the cytotoxic effect of recombinant
20 human TNF on WEHI-164 mouse fibrosarcoma cells in culture. One microgram of MAB 1 neutralizes approximately 156.25 units of TNF in vitro (Fig. 3).
4. MAb 1 neutralises the tumour regression activity of TNF in the following mouse tumour models in vivo;
25 WEHI-164 subcutaneous solid tumour, the Meth A subcutaneous solid tumour and the Meth A ascites tumour (Figs. 4, 5 and 9).
5. MAb1 prevents cerebral damage caused by human TNF in mice infected with malarial parasites.
- 30 6. In radioreceptor assays MAb 1 prevents binding of TNF to receptors on WEHI-164 cells (Table 3).
7. MAb 1 inhibits the induction of procoagulant activity (tissue factor) on cultured bovine aortic endothelial cells (Fig. 6).
- 35 8. MAb 1 reduces the uptake of ¹²⁵I fibrinogen into

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tumours of mice treated with TNF (Fig. 7).

9. Mab 1 competes for binding of ^{125}I TNF and thus shares an overlapping epitope with the following monoclonal antibodies: 21, 25, 32, 47, 54 and 37.
- 5 10. Mab 1 does not compete for binding of ^{125}I TNF with the following monoclonal antibodies: 11, 12, 42, 53, 31 and 20 (Fig. 8).

10

TABLE 3

RADIORECEPTOR ASSAY: INHIBITION OF TNF BINDING TO WEHI-164 CELLS BY Mab 1

15	TREATMENT	% SPECIFIC BINDING
	Mab 1 1/10	0
	1/100	21
	1/1,000	49
20	1/10,000	73
	1/100,000	105
	cold TNF (ng/tube)	
	10,000	0
	5,000	0
25	1,000	0
	500	10
	100	11
	10	64
	1	108
30	0	100

Mab 32 is an IgG2b,K antibody with an affinity for human TNF alpha of 8.77×10^{-9} moles/litre as determined by Scatchard analysis. This monoclonal antibody does not
 35 react with either human TNF beta (lymphotoxin) or mouse TNF alpha.

As shown in Figure 3 Mab 32 does not inhibit TNF cytotoxicity in vitro as determined in the WEHI-164 assay.

Monoclonal antibody 32 variably enhances TNF-induced
 40 tumour regression activity against WEHI-164 fibrosarcoma tumours implanted subcutaneously into BALB/c mice at a TNF dose of 10ug/day (see Figs. 10 and 11). This feature is not common to all monoclonal antibodies directed against

- 25 -

TNF (Fig. 9) but resides within the binding site specificity of MAb 32 (Fig. 8) which may allow greater receptor mediated uptake of TNF into tumour cells (see Table 4).

5

TABLE 4

BINDING OF TNF TO RECEPTORS ON WEHI-164 CELLS IN THE PRESENCE OF MAb 32

10

% BINDING¹²⁵ I-TNF

MAB DILUTION	CONTROL MAB	MAB 32
1/10	36	141
1/100	74	88
1/1000	101	83
15 1/10,000	92	82
1/100,000	97	93

Enhancement of TNF activity by MAb 32 at lower doses of TNF is such that at least tenfold less TNF is required to achieve the same degree of tumour regression (see Fig. 11 and 18). The results for day 1, 2.5ug and 1ug TNF and day 2, 5ug, 2.5ug and 1ug are statistically significant in a t-test at $p < .01$ level. This level of enhancement also increases the survival rate of recipients since the lower dose of TNF used is not toxic. Fig. 19 shows that univalent Fab fragments of MAb 32 also cause enhancement of TNF-induced tumour regression in the same manner as whole MAb 32 (see below).

MAb 32 inhibits the expression of clotting factors on endothelial cells normally induced by incubation of the cultured cells with TNF (see Fig. 6). This response may be mediated by a previously unidentified TNF receptor which is distinct to the receptor found on other cells.

Conversely, MAb 32 enhances the in vivo activation of coagulation within the tumour bed as shown by the

incorporation of radiolabelled fibrinogen (Fig. 7). This may be due to activation of monocytes/macrophage procoagulant and may provide further insight into the mechanism of TNF-induced tumour regression.

5 The results obtained with MAb 32 are shown in comparison to other anti-TNF MABs in Table 2.

10 The ability of MAB 32 and MAB 47 to inhibit the binding of TNF to endothelial cells was also assessed. Bovine aortic endothelial (BAE) cells (passage 11) were
15 plated in 24-well culture dishes (Corning) which had been pre-coated with gelatin (0.2%) and grown to confluence in McCoy's 5A (modified) medium supplemented with 20% foetal calf serum. For the radio-receptor assay all dilutions
20 (of cold TNF and MABs) were made in this medium. The BAE cells were incubated for one hour in the presence of either cold TNF (0 to 100ng) or MAB (ascites globulins diluted 1/100 to 1/100,000) and iodinated TNF (50,000 cpm). At the end of this time the medium was withdrawn and the cells washed before being lysed with 1M sodium hydroxide. The cell lysate was then counted for bound radioactive TNF. Specific binding of labelled TNF to the cells was then determined.

 The results obtained in this assay with MAB 32, MAB 47 and a control MAB are set out in Figure 12.

25 The results obtained in the clotting assay using BAE cells cultured in the presence of TNF and anti-TNF MAB correlate with the results obtained in the BAE radioreceptor assay i.e. MABs which inhibit the induction of clotting factors on the surface of endothelial cells
30 (as shown by the increase in clotting time compared to TNF alone) also inhibit the binding of TNF to its receptor. This is exemplified by MABs 32 and 47.

 MAB 32, which does not inhibit TNF binding to WEHI-164 cells, does inhibit binding of TNF to endothelial
35 cells. This result provides support for the hypothesis

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that distinct functional sites exist on the TNF molecule and that these sites interact with distinct receptor subpopulations on different cell types. Thus ligands which bind to defined regions of TNF are able to modify the biological effects of TNF by limiting its binding to particular receptor subtypes.

As shown in Figure 12 MAb 47 is a particularly potent inhibitor of TNF interaction with endothelial cells, the percentage specific binding at a dilution of 1/100 to 1/10,000 being effectively zero.

RECEPTOR BINDING STUDIES OF HUMAN TNF COMPLEXED WITH MAB 32 ON HUMAN CARCINOMA CELL LINES IN VITRO

Mab 32 has been shown to enhance the anti-tumour activity of human TNF. The mechanisms behind the enhancement may include restriction of TNF binding to particular (tumour) receptor subtypes but not others (endothelial) with subsequent decrease in TNF toxicity to non-tumour cells. This mechanism does not require enhanced uptake of TNF by tumour cells in in vitro assays. In addition, MAB 32 also potentiates the binding of human TNF directly to TNF receptors on certain human carcinoma cell lines.

MATERIALS AND METHODS

The following human carcinoma cell lines have been assayed for enhanced receptor-mediated uptake of TNF in the presence of MAB 32: B10, CaCo, HT 29, SKC01 (all colon carcinomas), 5637 (Bladder carcinoma), MM418E (melanoma), IGR3 (melanoma), MCF 7 (breast carcinoma). The cells were propagated in either RPMI-1640 (MM418E) DMEM (CaCo and IGR 3) or Iscoves modified DMEM (B10, HT 29, SK01, S637, MCF 7) supplemented with 10% foetal calf serum, penicillin/streptomycin and L-glutamine. Receptor assays were performed as previously described for endothelial cells except that the incubation time with iodinated TNF was extended to 3 hours for all but the B10

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cells for which the radiolabel was incubated for 1 hour.

RESULTS

Enhanced TNF uptake was observed in the presence of MAb32 by the melanoma cell lines tested MM418E and IGR 3 (Figs. 13 and 14), the bladder carcinoma 5637 (Fig. 15), and the breast carcinoma MCF 7 (Fig. 16). MAb 32 did not affect TNF-receptor interaction in any of the other cell lines as shown by B 10 (Fig. 17) MAb 47, which has been shown to inhibit TNF binding to WEHI-164 cells and endothelial cells, and which also inhibits TNF-mediated tumour regression was found to markedly inhibit TNF binding to all the cell lines tested (Figs. 13-17).

CONCLUSIONS

Receptor binding analyses have indicated a second mechanism whereby MAb 32 may potentiate the anti-tumour activity of TNF. This second pathway for enhancement of TNF results from increased uptake of TNF by tumour all receptors in the presence of MAb 32.

20 ENHANCEMENT OF TNF-MEDIATED TUMOUR REGRESSION IN VIVO BY MAB 32 OR UNIVALENT FAB' FRAGMENTS OF MAB 32

Tumour regression studies were carried out as described above in mice carrying WEHI-164 subcutaneous tumours (N = 5 animals/group). Tumour size was determined daily during the course of the experiment. The results obtained using MAb 32 are set out in Fig. 22 and show the mean \pm SD% change in tumour area at the completion of treatment (day 2) (■ MAb 32: □ control MAb: *MAb 47). Differences observed between control MAb-TNF and MAb 32-TNF treated groups are statistically significant in a T-test at the $p < .01$ level.

The results using the univalent Fab' fragments of MAb 32 are shown in Fig. 19. Tumour size was determined daily during the course of the experiment. The results show the mean SD% change in tumour area at the completion of

treatment (day 2). Differences between the control MAb-10F and MAb 32-TNF treated groups are statistically significant in a T-test at the $P < .01$ level.

TNF INDUCED TUMOUR REGRESSION : EFFECT OF ANTI-PEPTIDE 301

5 SERA

Fig. 20 shows the percent change in tumour area in tumour-bearing mice treated for three days with TNF plus control MAb (antibody against bovine growth hormone), TNF plus MAb 32 or TNF plus antiserum (globulin fraction) against peptide 301. In an unpaired T-test the control group is significantly different from both of the test groups (MAb 32, antiserum 301) while the MAb 32 and peptide antiserum 301 groups are not significantly different from each other. (control vs MAb 32, $p < .002$; control vs antipeptide 301, $p < .025$). Thus antisera raised using a peptide which comprises part of the MAb 32 specificity, also causes TNF enhancement of tumour regression.

As shown in Fig. 9 competition binding studies has shown that the thirteen monoclonal antibodies can be sub-divided into two main groups, namely MAb 1, 21, 47, 54, 37, 32 and 25 and MAb 11, 12, 53 and 42. Experiments were then conducted to identify the regions on human TNF recognised by these monoclonal antibodies.

25 IDENTIFICATION OF REGIONS ON HUMAN TNF RECOGNISED BY MONOCLONAL ANTIBODIES

Methods

1. Overlapping peptides of 7 and 10 amino acid residues long were synthesized on polypropylene pins according to the method of Geysen et al., 1984, PNAS 81, 3998-4002. The overlap was of 6 and 9 residues respectively and collectively the peptides covered the entire TNF amino acid sequence. The peptides were tested for reactivity with the MAb by ELISA. MAb which had TNF reactivity absorbed from them by prior incubation with whole TNF were

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also tested for reactivity with the peptides and acted as a negative control.

2. Longer peptides of TNF were synthesized as described below. These peptides were used to raise antisera in sheep using the following protocol. Merino sheep were primed with TNF peptide conjugated to ovalbumin and emulsified in Freund's Complete adjuvant and boosted at 4 weekly intervals with peptide-ovalbumin and sera assayed for the presence of anti-TNF antibody by radioimmunoassay. Of the peptides shown only peptides 275, 301, 305, 306 and 307 elicited sera reacting with whole TNF. The positive sera were then used in competitive binding assays (PACT assays) with the MABs.

- The following peptides were synthesised and are described using the conventional three letter code for each amino acid with the TNF sequence region indicated in brackets.

Peptide 275

H-Ala-Lys-Pro-Trp-Tyr-Glu-Pro-Ile-Tyr-Leu-OH (111-120)

20 Peptide 301

H-Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala-OH (1-18)

Peptide 302

- 25 H-Leu-Arg-Asp-Asn-Gln-Leu-Val-Val-Pro-Ser-Glu-Gly-Leu-Tyr-Leu-Ile-OH (43-58)

Peptide 304

H-Leu-Phe-Lys-Gly-Gln-Gly-Cys-Pro-Ser-Thr-His-Val-Leu-Leu-Thr-His-Thr-Ile-Ser-Arg-Ile-OH (63-83)

Peptide 305

- 30 H-Leu-Ser-Ala-Glu-Ile-Asn-Arg-Pro-Asp-Tyr-Leu-Asp-Phe-Ala-Glu-Ser-Gly-Gln-Val-OH (132-150)

Peptide 306

H-Val-Ala-His-Val-Val-Ala-Asn-Pro-Gln-Ala-Glu-Gly-Gln-Leu-OH (13-26)

- 35 Peptide 307

H-Ala-Glu-Gly-Gln-Leu-Gln-Trp-Leu-Asn-Arg-Arg-Ala-Asn-Ala-Leu-Leu-Ala-Asn-Gly-OH (22-40)

Peptide 308

5 H-Gly-Leu-Tyr-Leu-Ile-Tyr-Ser-Gln-Val-Leu-Phe-Lys-Gly-Gln-Gly-OH (54-68)

Peptide 309

H-His-Val-Leu-Leu-Thr-His-Thr-Ile-Ser-Arg-Ile-Ala-Val-Ser-Thr-Gln-Thr-Lys-Val-Asn-Leu-Leu-COOH (73-94)

Peptide 323

10 H-Thr-Ile-Ser-Arg-Ile-Ala-Val-Ser-Thr-Gln-Thr-OH (79-89)

These peptides were synthesised using the following general protocol.

All peptide were synthesised using the Fmoc-polyamide method of solid phase peptide synthesis (Atherton et al, 15 1978, J.Chem.Soc.Chem.Comm., 13, 537-539). The solid resin used was PepSyn KA which is a polydimethylacrylamide gel on Kieselguhr support with 4-hydroxymethylphenoxy-acetic acid as the functionalised linker (Atherton et al., 1975, J.Am.Chem. Soc. 97, 6584-6585).

20 The carboxy terminal amino acid was attached to the solid support by a DCC/DMAP-mediated symmetrical-anhydride esterification.

All Fmoc-groups were removed by piperidine/DMF wash and peptide bonds were formed either via pentafluorophenyl 25 active esters or directly by BOP/MMM/HOBt (Castro's reagent) (Fournier et al, 1989, Int.J.Peptide Protein Res., 33, 133-139) except for certain amino acids as specified in Table 5.

30 Side chain protection chosen for the amino acids was removed concomittantly during cleavage with the exception of Acn on cysteine which was left on after synthesis.

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TABLE 5

	<u>Amino Acid</u>	<u>Protecting Group</u>	<u>Coupling Method</u>
	Arg	Mtr or Pmc	Either
	Asp	OBu	Either
	Cys	Acm (permanent)	Either
	Glu	OBu	Either
	His	Boc	OPfp only
	Lys	Boc	Either
5	Ser	But	BOP only
	Thr	But	BOP only
	Tyr	But	Either
	Trp	none	Either
	Asn	none	OPfp only
10	Gln	none	OPfp only

Cleavage and Purification

Peptide 301, 302, 305 are cleaved from the resin with 95% TFA and 5% thioanisole (1.5 h) and purified on reverse phase C4 column, (Buffer A - 0.1% aqueous TFA, Buffer B - 80% ACN 20% A).

Peptide 303, 304 are cleaved from the resin with 95% TFA and 5% phenol (5-6 h) and purified on reverse phase C4 column. (Buffers as above).

Peptide 306, 308 are cleaved from the resin with 95% TFA and 5% water (1.5 h) and purified on reverse phase C4 column. (Buffers as above).

Peptide 309 Peptide was cleaved from the resin with 95% TFA and 5% thioanisole and purified on reverse phase C4 column. (Buffers as above).

Peptide 307 Peptide was cleaved from the resin with a mixture of 93% TFA, 3.1% Anisole, 2.97% Ethylmethylsulfide and 0.95% Ethanedithiol (3 h) and purified on reverse phase C4 column. (Buffers as above).

RESULTS

Typical results of MAb ELISA using the 7 and 10 mers are shown in Fig. 21. Together with the results of PACT

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assays using the sheep anti-peptide sera (shown in Table 6) the following regions of TNF contain the binding sites of the anti-TNF MAb.

- Mab 1 : residues 1-18, 58-65, 115-125, 138-149
- 5 Mab 11: residues 49-98
- Mab 12: residues 22-40, 70-87
- Mab 21: residues 1-18, 76-90
- Mab 25: residues 12-22, 36-45, 96-105, 132-157
- Mab 32: residues 1-26, 117-128, 141-153
- 10 Mab 37: residues 22-31, 146-157
- Mab 42: residues 22-40, 49-96, 110-127, 136-153
- Mab 47: residues 1-18, 108-128
- Mab 53: residues 22-40, 69-97, 105-128, 135-155
- Mab 54: residues 56-79, 110-127, 136-155

15

TABLE 6
COMPETITIVE BINDING OF TNF BY ANTI-TNF MONOCLONES
IN THE PRESENCE OF ANTI PEPTIDE SERA

20	<u>MAB/PEPTIDE SERA</u>				
	275	301	305	306	307
	1	+	-	-	-
	11	+/-	-	-	-
	12	+	-	-	++
25	21	++	-	-	-
	25	+	-	-	-
	32	++++	+	+	-
	37	+	+/-	-	+
	47	+	-	-	-
30	53	+	-	-	+
	54	+	-	-	-
	42	+	+	-	+

- Note 1: - indicates no competition, + indicates slight competition at high concentration of anti-peptide antisera (1/50), ++++ indicates strong competition by anti-peptide sera equal to that of the homologous MAb.
- 35 Note 2: Only peptide which elicited sera recognising whole TNF were used in this assay.

CONCLUSIONS

Mapping of the regions recognised by each of the MABs has indicated that MABs in group I (MABs 1, 21, 47, 54, 37, 32 and 25) as shown on the schematic diagram bind TNF in the region of residues 1-18 with the exception of MABs 37 and 54, while MABs in group II of the schematic diagram (MABs 11, 12, 53 and 42) bind TNF in the region of residues 70-96 which encompasses a so-called pallendromic loop on the TNF 3-D structure. MABs which inhibit the induction of endothelial cell procoagulant activity (MABs 1, 32, 42, 47, 54 and 53) all bind in the region of residues 108-128 which again contains a loop structure in the 3-D model and may indicate that this region interacts with TNF receptors which are found on endothelial cells but not tumour cells. MAB 32 which potentiates the in vivo tumour regression and anti-viral activity of TNF is the only antibody which binds all the loop regions associated with residues 1-26, 117-128, and 141-153 and hence binding of these regions is crucial for enhanced TNF bioactivity with concomittant reduction of toxicity for normal cells.

As is apparent from Table 2 MAB 1, 47 and 54 have the same effect on the bioactivity of TNF. From the results presented above it is noted that these three monoclonals bind to similar regions of the TNF molecule. Accordingly, it is believed that a ligand which binds to TNF in at least two regions selected from the group consisting predominately of the region of residues 1-20, the region of residues 56-77, the region of residues 108-128 and the region of residues 138-149 will effect the bioactivity of TNF in a manner similar to that of MABs 1, 47 and 54. Similarly, it is believed that a ligand which binds to TNF predominately in the regions of residues 1-20 and 76-90 will have the same effect on the bioactivity of TNF as MAB 21. A ligand which binds to TNF predominately in the

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regions of residues 22-40 and 69-97 will have the same effect on bioactivity of TNF as MAb 12. A ligand which binds to TNF predominately in the regions of residues 1-30, 117-128, and 141-153 would be expected to have the same effect on the bioactivity of TNF as MAb 32 and a ligand which binds to TNF predominately in the regions of residues 22-40, 49-97, 110-127 and 136-153 would be expected to have the same effect on the bioactivity of TNF as MAb 42. A ligand which binds to TNF predominately in the regions of residues 22-31 and 146-157 would be expected to have the same effect on the bioactivity of TNF as MAb 37 and a ligand which binds to TNF predominately in the regions of residues 22-40, 69-97, 105-128 and 135-155 would be expected to have the same effect on the bioactivity of TNF as MAb 53.

The present inventors have quite clearly shown that the bioactivity of TNF can be altered by the binding of a ligand to the TNF, and that the effect on the bioactivity is a function of the specificity of the ligand. For example, the binding of MAb 32 to TNF in the regions of residues 1-26, 117-128 and 141-153 results in the induction of endothelial procoagulant activity of the TNF and binding of TNF to receptors on endothelial cells being inhibited; the induction of tumour fibrin deposition and tumour regression activities of the TNF being enhanced; the cytotoxicity being unaffected and the tumour receptor binding activities of the TNF being unaffected or enhanced. It is believed that this effect on the bioactivity of the TNF may be due to the prevention of the binding of the epitope of the TNF recognised by MAb 32 to naturally occurring biologically active ligands. Accordingly, it is believed that a similar effect to that produced by MAb 32 could also be produced by a ligand which binds to a region of TNF in a manner such that the epitope recognised by MAb 32 is prevented from binding to

naturally occurring biologically active ligands. This prevention of binding may be due to steric hindrance or other mechanisms.

Accordingly, it is intended that the prevention of
5 the binding of epitopes recognised by the various monoclonal antibodies described herein to naturally occurring biologically active ligands is within the scope of the present invention.

CLAIMS:-

1. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the following biological activities of the TNF are inhibited:-
 1. Tumour regression;
 2. Induction of endothelial procoagulant;
 3. Induction of tumour fibrin deposition;
 4. Cytotoxicity; and
 5. Receptor binding.
2. A ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the induction of endothelial procoagulant, tumour regression, induction of tumour fibrin deposition, cytotoxicity and receptor binding activities of the TNF are inhibited, the ligand binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 1-18, 58-65, 115-125 and 138-149, or the topographic region of residues 1-18 and 108-128, or the topographic region of residues 56-79, 110-127 and 135-155 is substantially prevented from binding to naturally occurring biologically active ligands.
3. A ligand which binds to human TNF in at least two regions selected from the group consisting of the topographic region of residues 1-20, the topographic region of residues 56-77, the topographic region of residues 108-127 and the topographic region of residues 138-149.
4. A ligand as claimed in claim 2 in which the ligand binds to human TNF in the topographic regions of residues 1-18, 58-65, 115-125 and 138-149.
5. A ligand as claimed in claim 2 in which the ligand binds to human TNF in the topographic regions of residues 1-18, and 108-128.
6. A ligand as claimed in claim 2 in which the ligand binds to human TNF in the topographic regions of residues 56-79, 110-127 and 136-155.
7. A ligand as claimed in any one of claims 1 to 6 in which the ligand is selected from the group consisting of

- antibodies, F(ab) fragments, single domain antibodies (dAbs) restructured antibodies, single chain antibodies and serum binding proteins.
8. A ligand as claimed in claim 7 in which the ligand is a monoclonal antibody or F(ab) fragment thereof.
9. A ligand as claimed in any one of claims 1, 2, 3, 6 or 7 in which the ligand is a monoclonal antibody selected from the group consisting of MAb 1 (ECACC 89080301), MAb 54 (ECACC 89083103) and MAb 47 (ECACC 89121402).
10. A ligand as claimed in claim 4 in which the ligand is MAb 1 (ECACC 89080301).
11. A ligand as claimed in claim 5 in which the ligand is MAb 54 (ECACC 89083103).
12. A ligand as claimed in claim 6 in which the ligand is MAb 47 (ECACC 89121402).
13. A composition comprising TNF in combination with a ligand as claimed in any one of claims 1 to 12, the ligand being bound to the TNF.
14. A method of treating toxic shock comprising administering either the ligand as claimed in any one of claims 1 to 12 or the composition as claimed in claim 12.
15. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited; the induction of tumour fibrin deposition and tumour regression activities of the TNF are enhanced; and the cytotoxicity and receptor binding activities of the TNF are unaffected.
16. A ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited; the binding of the TNF to receptors on endothelial cells is inhibited, the induction of tumour fibrin deposition and tumour regression activities of the TNF are enhanced; and the cytotoxicity and tumour receptor

binding activities of the TNF are unaffected or enhanced; the ligand binding to the TNF such that the epitope of the TNF defined by the topographic region of residues 1-30, 117-128 and 141-153 is substantially prevented from binding to naturally occurring biologically active ligands.

17. A ligand which binds to human TNF in the topographic regions of residues 1-30, 117-128 and 141-153.

18. A ligand as claimed in claim 17 in which the ligand binds to human TNF in the topographic regions of residues 1-26, 117-128 and 141-153.

19. A ligand as claimed in any one of claims 14 to 16 in which the ligand is selected from the group consisting of antibodies, F(ab) fragments, single domain antibodies (dABs) restructured antibodies, single chain antibodies and serum binding proteins.

20. A ligand as claimed in claim 19 in which the ligand is a monoclonal antibody or F(ab) fragment thereof.

21. A ligand as claimed in any one of claims 15 to 20 in which the ligand is MAb 32 (ECACC 89080302).

22. A composition comprising TNF in combination with a ligand as claimed in any one of claims 15 to 21 in which the ligand is bound to the TNF.

23. A method of treating tumours the growth of which is inhibited by TNF, the method comprising administering either the ligand of any one of claims 15 to 21 or the composition of claim 22.

24. A method as claimed in claim 23 in which the tumour is selected from the group consisting of melanoma, breast and bladder carcinomas.

25. A method as claimed in claim 23 or 24 in which the method comprises the co-administration of a cytotoxic drug used in cancer therapy.

26. A method as claimed in claim 24 in which the cytotoxic drug is selected from the group consisting of vinblastin, acyclovir, interferon alpha, IL-2, actinomycin

D, AZT, radiotherapy, adriamycin, mytomycin C, cytosine arabinoside, dounorubicin, cis-platin, vincristine, 5-flurouracil and bleomycin.

27. A ligand which binds to residues 1-18 of human TNF (peptide 301).

28. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited; the binding of TNF to receptors on endothelial cells is inhibited; the induction of tumour fibrin deposition and tumour regression activities of the TNF are enhanced; the cytotoxicity of the TNF is unaffected and the tumour receptor binding activities of the TNF are unaffected or enhanced, the ligand binding to TNF such that the epitope of the TNF defined by the topographic region of residues 1-18 is substantially prevented from binding to naturally occurring biologically active ligands.

29. A composition comprising TNF in combination with a ligand as claimed in claim 27 or 28 in which the ligand is bound to the TNF.

30. A method of treating tumours the growth of which is inhibited by TNF, comprising administering either the ligand as claimed in claim 27 or 28 or the composition of claim 29.

31. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the cytotoxicity and tumour regression activities of the TNF are unaffected and the induction of endothelial procoagulant, induction of fibrin deposition and receptor binding activities of the TNF are inhibited.

32. A ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the cytotoxicity and tumour regression activities of the TNF are unaffected; the induction of endothelial procoagulant and induction of tumour fibrin deposition activities of the

TNF are inhibited and the receptor binding activities of the TNF are unaffected, the ligand binding to TNF such that the epitope of the TNF defined by the topographic regions of residues 22-40, 49-97, 110-127 and 136-153 is substantially prevented from binding to naturally occurring biologically active ligands.

33. A ligand which binds to human TNF in the topographic regions of residues 22-40, 49-97, 110-127 and 136-153.

34. A ligand as claimed in claim 23 in which the ligand binds to human TNF in the topographic regions of residues 22-40, 49-96, 110-127 and 136-153.

35. A ligand as claimed in any one of claims 31 to 35 in which the ligand is selected from the group consisting of antibodies, F(ab) fragments, single domain antibodies (dABs) restructured antibodies, single chain antibodies and serum binding proteins.

36. A ligand as claimed in claim 35 in which the ligand is a monoclonal antibody or F(ab) fragment thereof.

37. A ligand as claimed in any one of claims 31 to 36 in which the ligand is MAb 42 (ECACC 89080304).

38. A composition comprising TNF in combination with a ligand as claimed in any one of claims 31 to 37 in which the ligand is bound to the TNF.

39. A method of treating tumours inhibited by the action of TNF comprising administering the ligand as claimed in any one of claims 31 to 37 or the composition of claim 38.

40. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the tumour fibrin deposition activity of the TNF is enhanced; the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity, tumour regression and receptor binding activities of the TNF are inhibited.

41. A ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the tumour fibrin deposition activity of the TNF is enhanced;

the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity, tumour regression and receptor binding activities of the TNF are inhibited, the ligand binding to TNF such that the epitope of the TNF defined by the topographic regions of residues 12-22, 36-45, 96-105 and 132-157 is substantially prevented from binding to naturally occurring biologically active ligands.

42. A ligand which binds to human TNF in the topographic regions of residues 12-22, 36-45, 96-105 and 132-157.

43. A ligand as claimed in any one of claims 40 to 42 in which the ligand is selected from the group consisting of antibodies, F(ab) fragments, single domain antibodies (dABs) restructured antibodies, single chain antibodies and serum binding proteins.

44. A ligand as claimed in claim 43 in which the ligand is a monoclonal antibody of F(ab) fragment thereof.

45. A ligand as claimed in any one of claims 40 to 44 in which the ligand is MAb 25 (ECACC 89121401).

46. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the tumour fibrin deposition activity of the TNF is enhanced and the cytotoxicity, tumour regression, induction of endothelial procoagulant and receptor binding activities of the TNF are inhibited.

47. A ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the tumour fibrin deposition activity of the TNF is enhanced and the cytotoxicity, tumour regression, induction of endothelial procoagulant and receptor binding activities of the TNF are inhibited, the ligand binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 1-20 and 76-90 is substantially prevented from binding to naturally occurring biologically active ligands.

48. A ligand which binds to human TNF in the topographic regions of residues 1-20 and 76-90.

49. A ligand as claimed in claim 48 in which the ligand binds to TNF in the regions of residues 1-18 and 76-90.
50. A ligand as claimed in any one of claims 46 to 49 in which the ligand is selected from the group consisting of antibodies, F(ab) fragments, single domain antibodies (dABs) restructured antibodies, single chain antibodies and serum binding proteins.
51. A ligand as claimed in claim 50 in which the ligand is a monoclonal antibody or F(ab) fragment thereof.
52. A ligand as claimed in any one claims 46 to 51 in which the ligand is MAb 21 (ECACC 90012432).
53. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the tumour fibrin deposition activity of the TNF is unaffected and the cytotoxicity, tumour regression, induction of endothelial procoagulant and receptor binding activities of the TNF are inhibited.
54. A ligand capable of binding to human TNF, the ligand being characterized in that when it binds to TNF the tumour fibrin deposition activity of the TNF is unaffected and the cytotoxicity, tumour regression, induction of endothelial procoagulant and receptor binding activities of the TNF are inhibited, the ligand binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 22-40, 69-97, 105-128 and 135-155 is substantially prevented from binding to naturally occurring biologically active ligands.
55. A ligand which binds to human TNF in the topographic regions of residues 22-40, 69-97, 105-128 and 135-155.
56. A ligand as claimed in any one of claims 53 to 55 in which the ligand is selected from the group consisting of antibodies, F(ab) fragments, single domain antibodies (dABs) restructured antibodies, single chain antibodies and serum binding proteins.
57. A ligand as claimed in claim 56 in which the ligand

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is a monoclonal antibody or F(ab) fragment thereof.

58. A ligand as claimed in any one of claims 53 to 57 in which the ligand is MAb 53 (ECACC 90012433).

59. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF fibrin deposition, induction of endothelial procoagulant, cytotoxicity, tumour regression and receptor binding activities of the TNF are unaffected.

60. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the tumour fibrin deposition, induction of endothelial procoagulant, cytotoxicity, tumour regression and receptor binding activities of the TNF are unaffected, the ligand binding to TNF such that the epitope of the TNF defined by the topographic regions of residues 22 - 31 and 146 - 157 is substantially prevented from binding to naturally occurring biologically active ligands.

61. A ligand which binds to human TNF in the topographic regions of residues 22-31 and 146-157.

62. A ligand as claimed in in any one of claims 59 to 61 in which the ligand is selected from the group consisting of antibodies, F(ab) fragments, single domain antibodies (dABs) restructured antibodies, single chain antibodies and serum binding proteins.

63. A ligand as claimed in claimed in claim 62 in which the ligand is a monoclonal antibody or F(ab) fragment thereof.

64. A ligand as claimed in any one of claims 59 to 63 in which the ligand is MAb 37 (ECACC 89080303).

65. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of procoagulant activity of the TNF is unaffected and the cytotoxicity, tumour regression, fibrin deposition and receptor binding activities of the TNF are inhibited.

66. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity, tumour regression, tumour fibrin deposition and receptor binding activities of the TNF are inhibited, the ligand binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 22 - 40 and 49 - 98 is substantially prevented from binding to naturally occurring biologically active ligands.

67. A ligand which binds to human TNF in at least one of the regions selected from the group consisting of the topographic region of residues 22-40, the topographic region of residues 49-98 and the topographic region of residues 69-97.

68. A ligand as claimed in claim 67 in which the ligand binds to human TNF in the topographic region of residues 49-98.

69. A ligand as claimed in claim 67 in which the ligand binds to human TNF in the topographic regions of residues 22-40 and 70-87.

70. A ligand as claimed in any one of claims 65 to 69 in which the ligand is selected from the group consisting of antibodies, F(ab) fragments, single domain antibodies (dABs) restructured antibodies, single chain antibodies and serum binding proteins.

71. A ligand as claimed in claim 70 in which the ligand is a monoclonal antibody or F(ab) fragment thereof.

72. A ligand as claimed in any one of claims 65, 66, 67, 70 and 71 in which the ligand is MAb 11 or MAb 12.

73. A ligand as claimed in claim 68 in which the ligand is MAb 11.

74. A ligand as claimed in claim 69 in which the ligand is MAb 12.

75. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF

is inhibited.

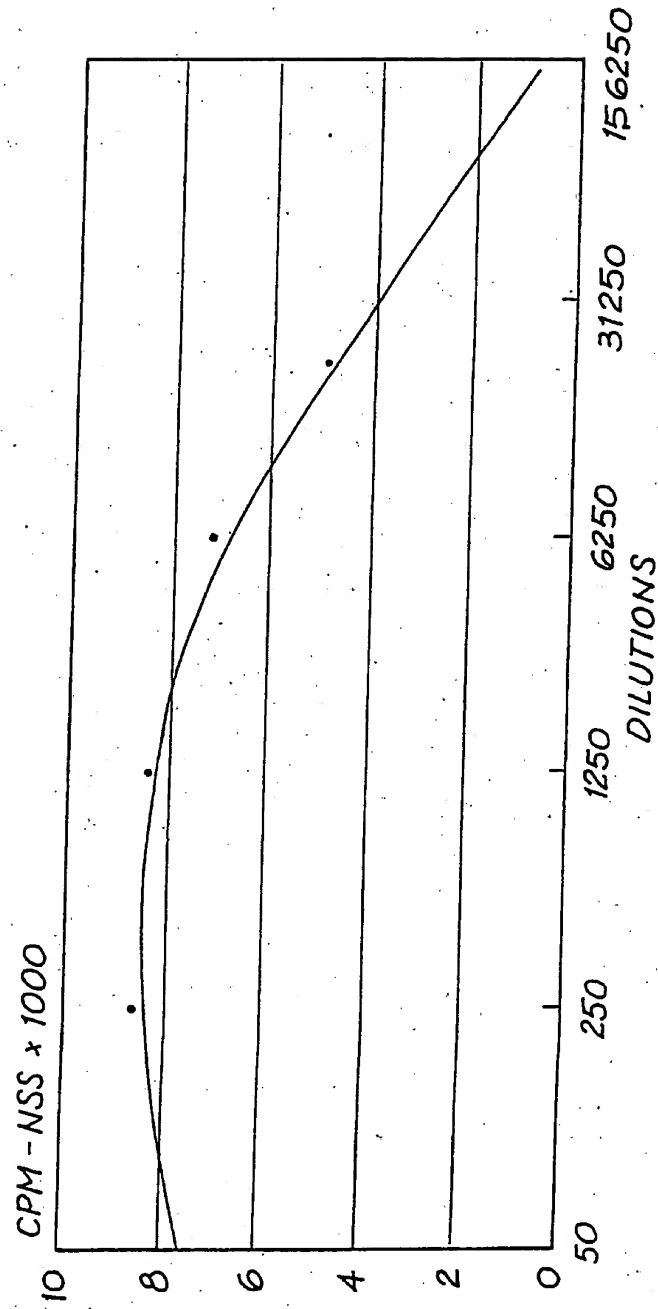
76. A ligand capable of binding to human TNF, the ligand being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited, the ligand binding to TNF such that the epitope of the TNF defined by the topographic region of residues 108 - 128 is prevented from binding to naturally occurring biologically active ligands.

77. A ligand which binds to human TNF in the region of residues 108 - 128.

78. A ligand as claimed in any one of claims 75 to 77 in which the ligand is selected from the group consisting of monoclonal antibodies designated MAb 1, MAb 32, MAb 42, MAb 47, MAb 53 and MAb 54.

79. A ligand which binds to TNF either in the region(s) shown topographically in any one of Figures 23 - 33 or binds to TNF in a manner such that the region(s) shown topographically in any one of Figures 23 - 33 is prevented from binding to naturally occurring biologically active ligands.

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TNF MAb 001
TITRE ASSAY

LEGEND: --- TNF 001

FIG. 1

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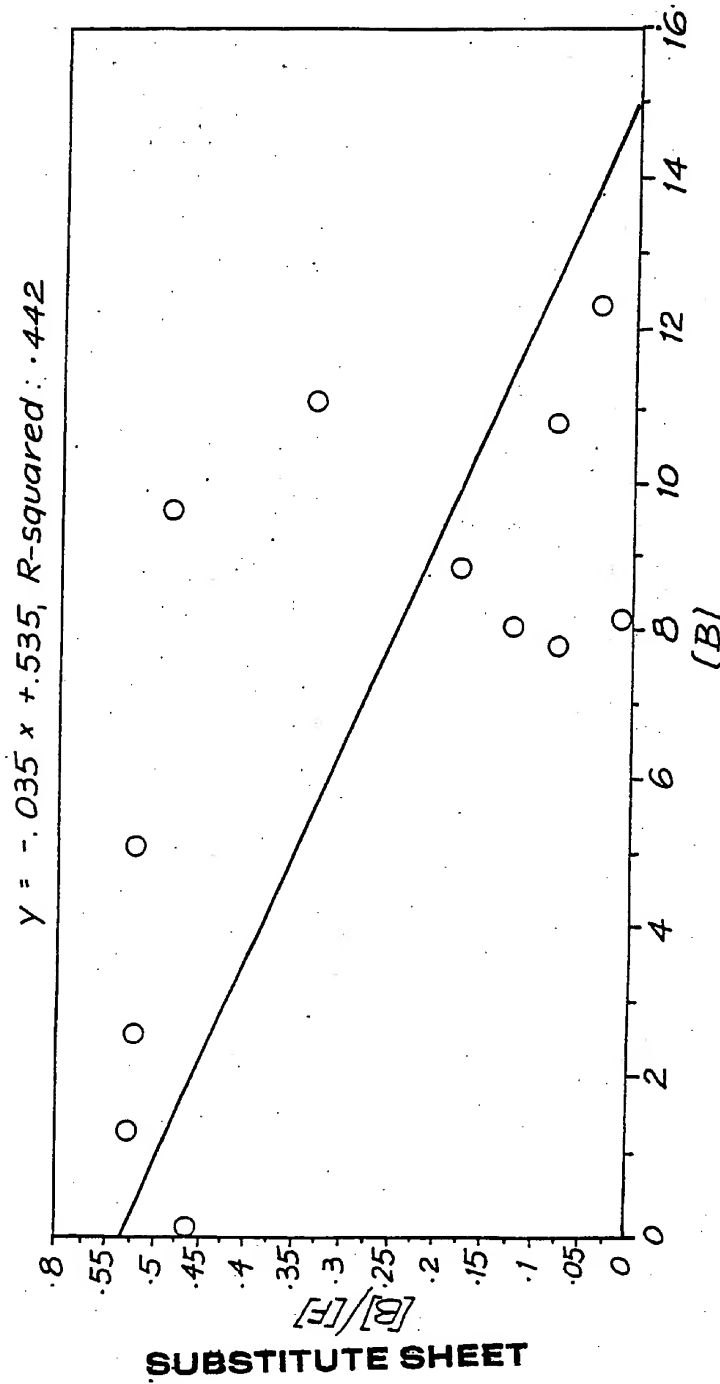


FIG. 2

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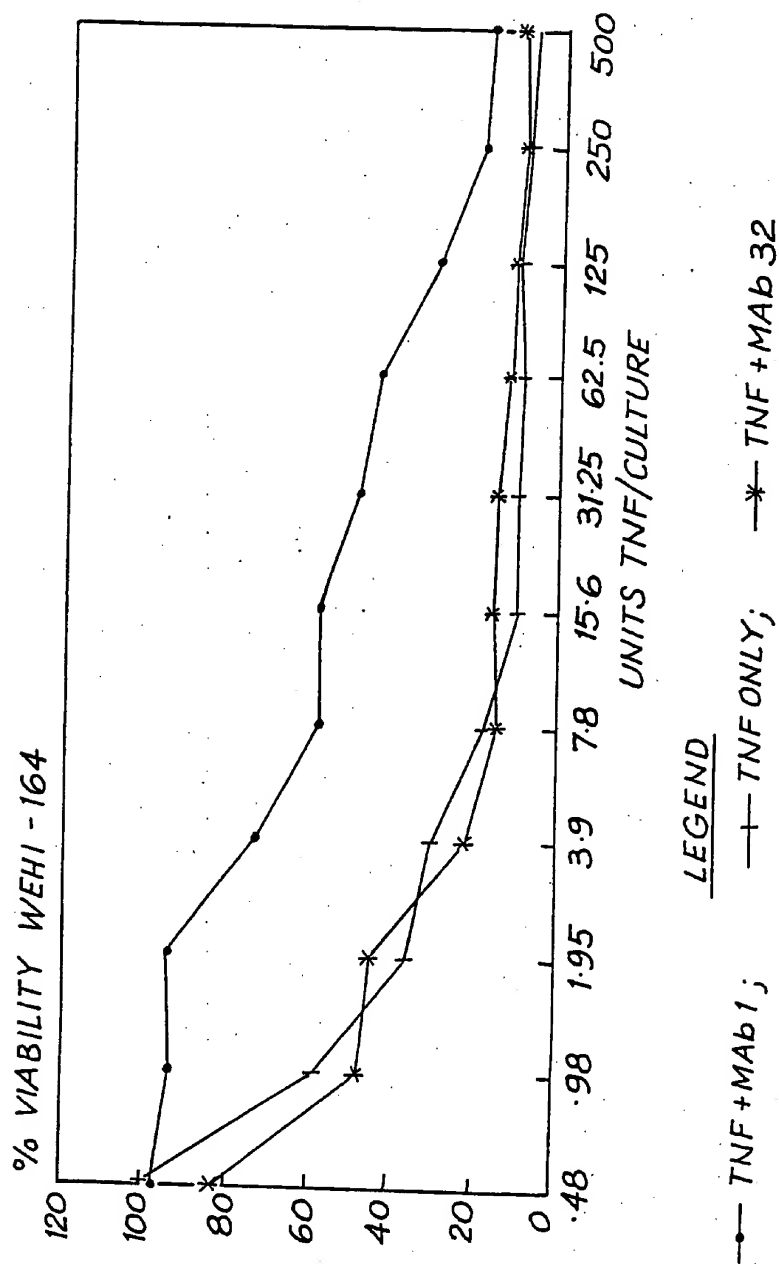


FIG. 3

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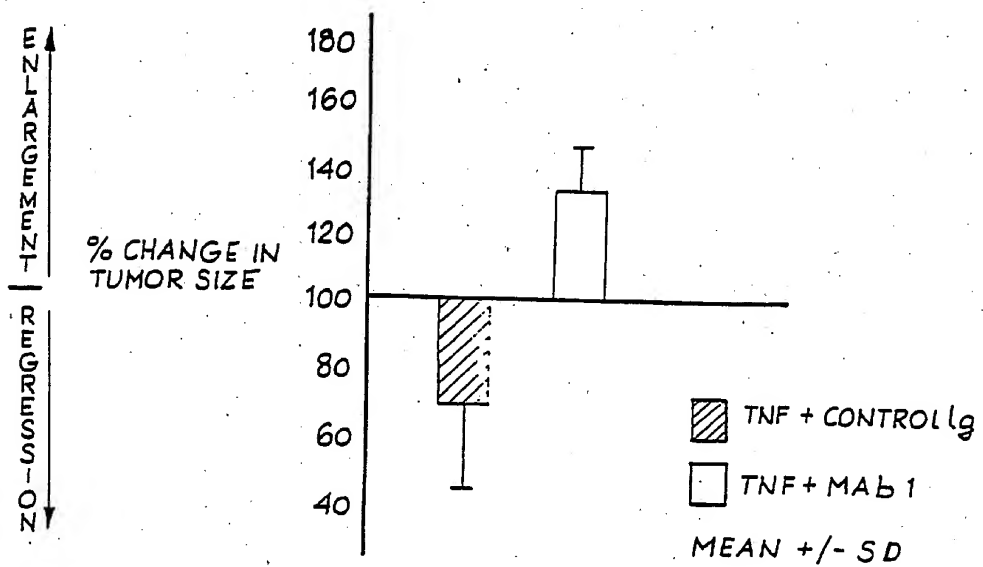


FIG. 4

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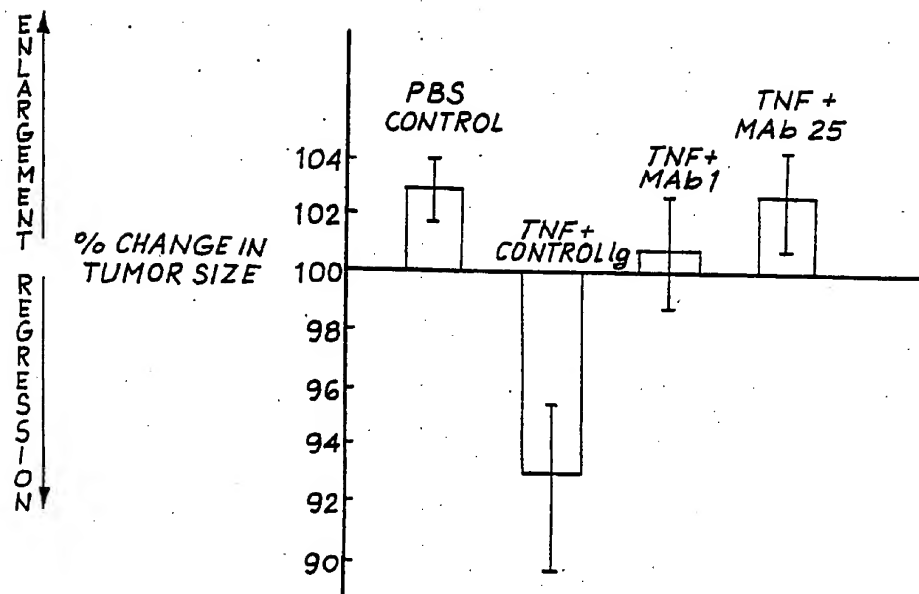


FIG. 5

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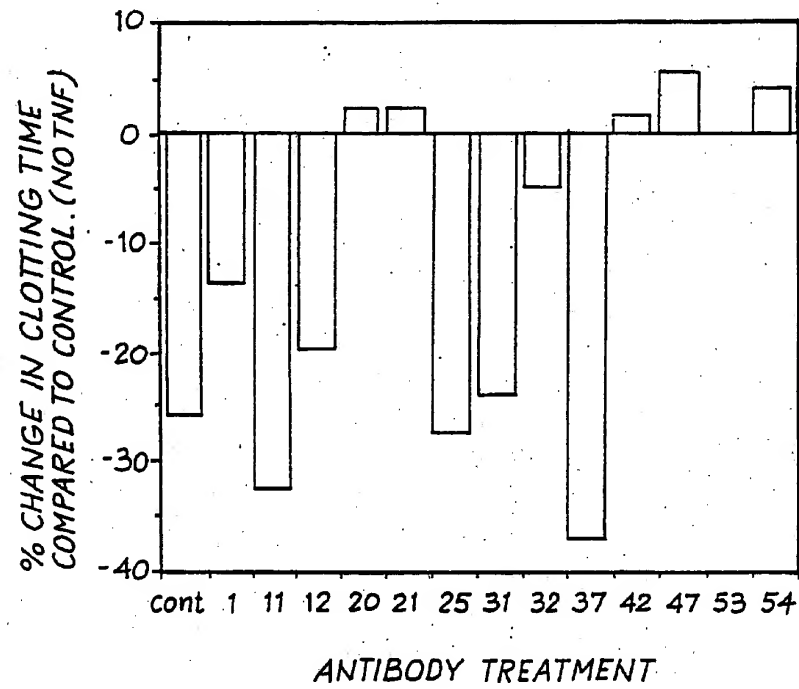


FIG. 6

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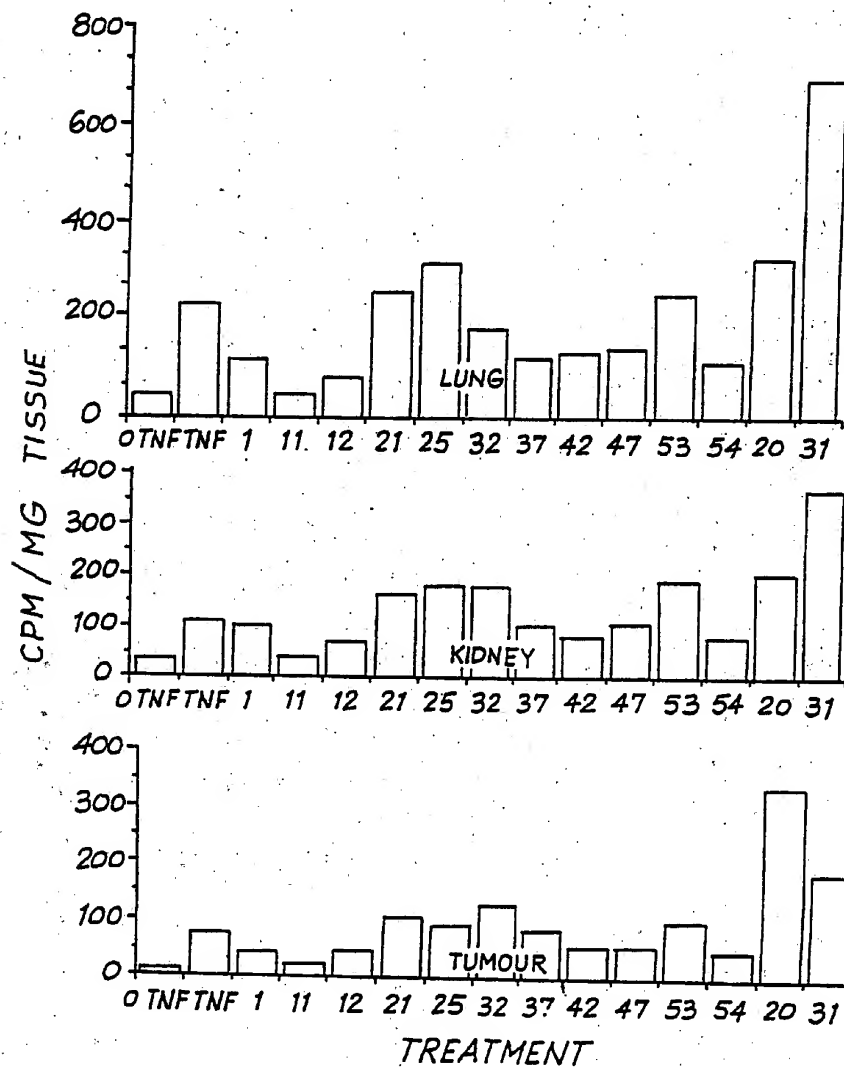


FIG. 7

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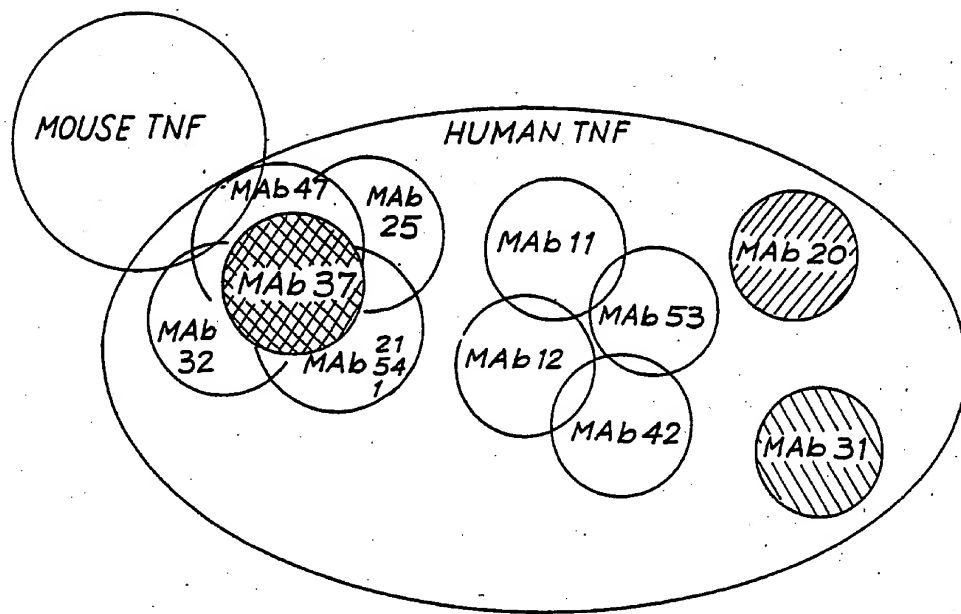


FIG. 8

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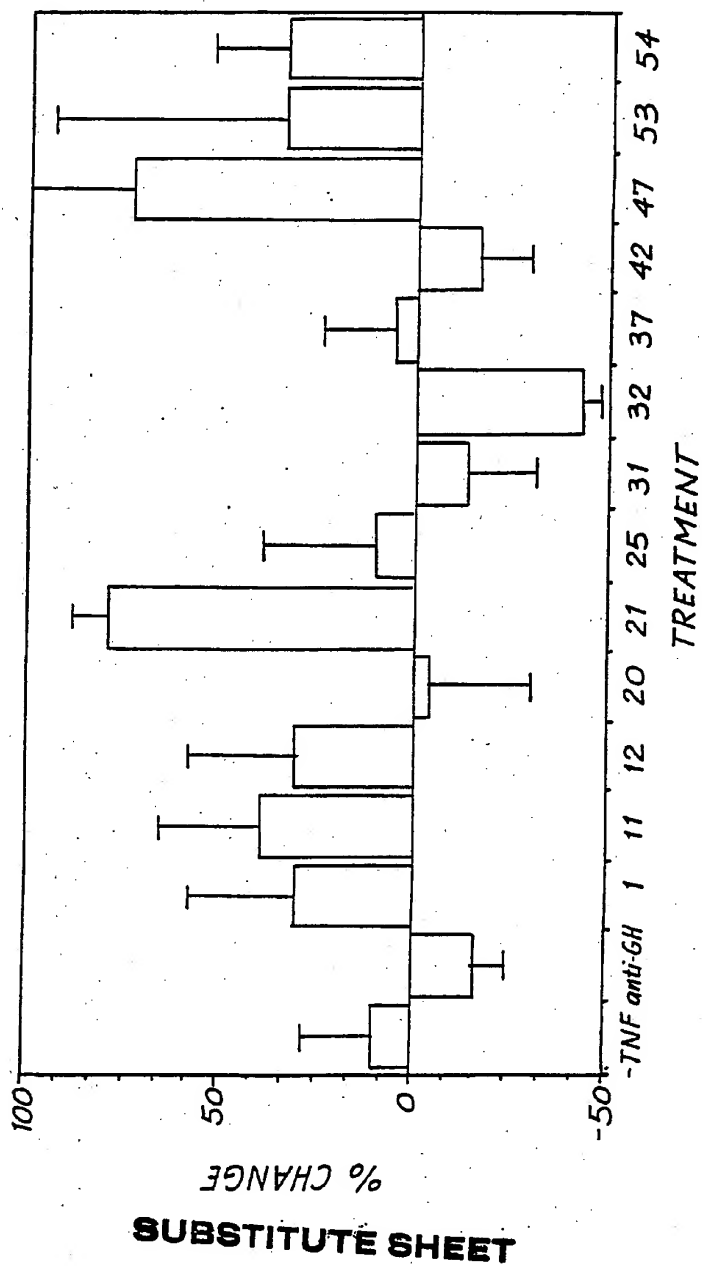
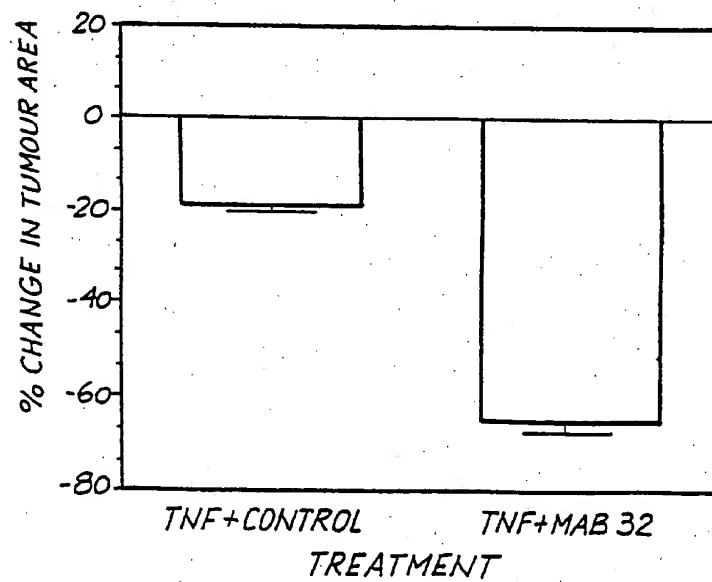
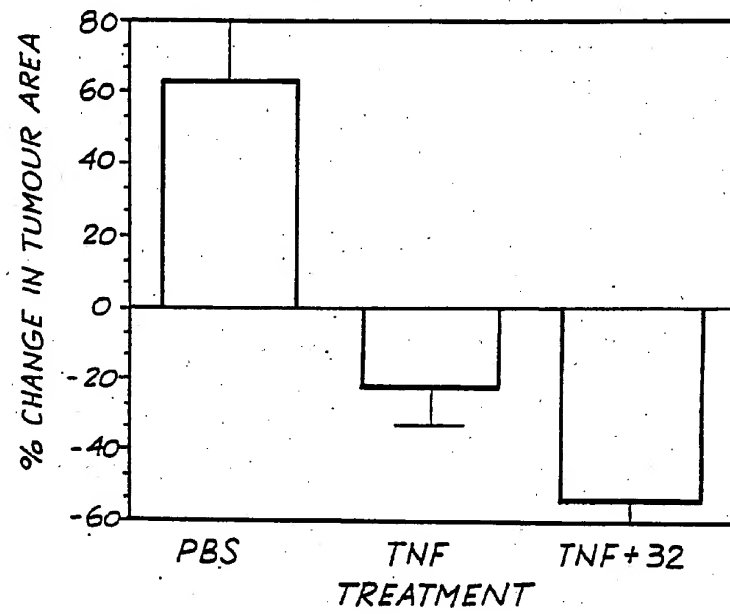


FIG. 9

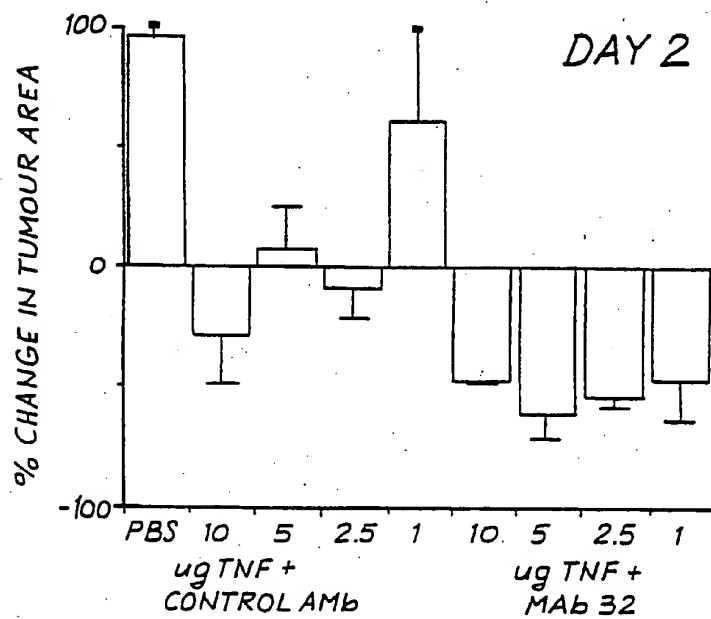
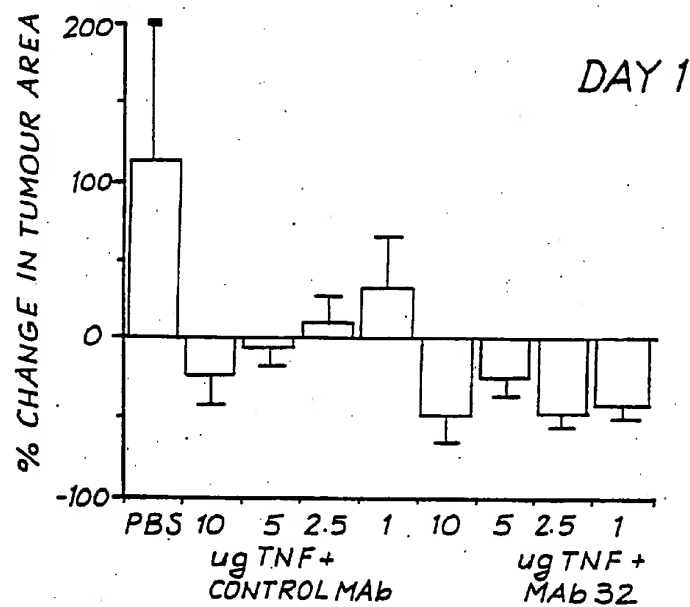
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FIG. 10

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FIG. 11



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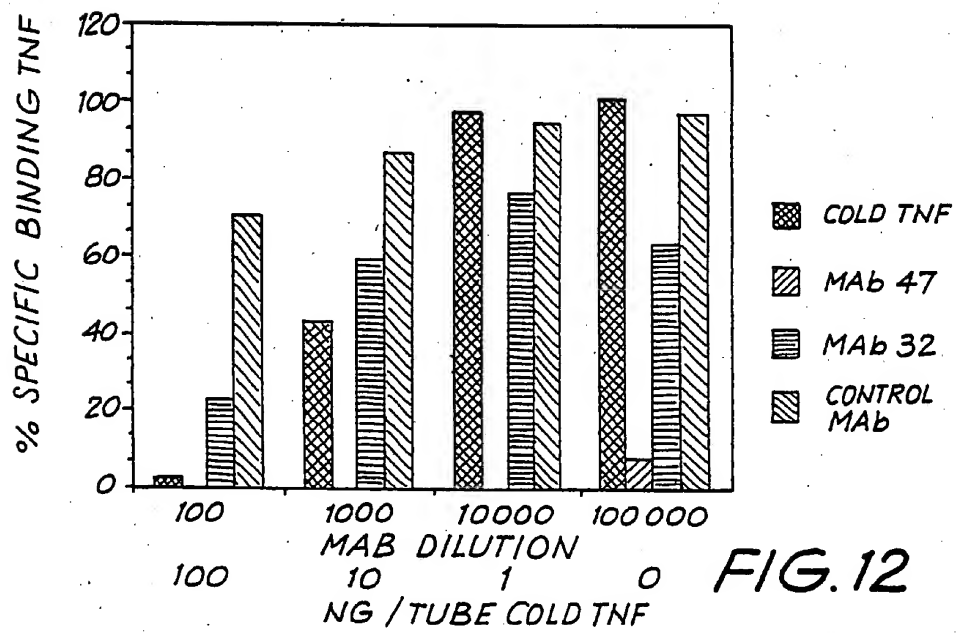


FIG.12

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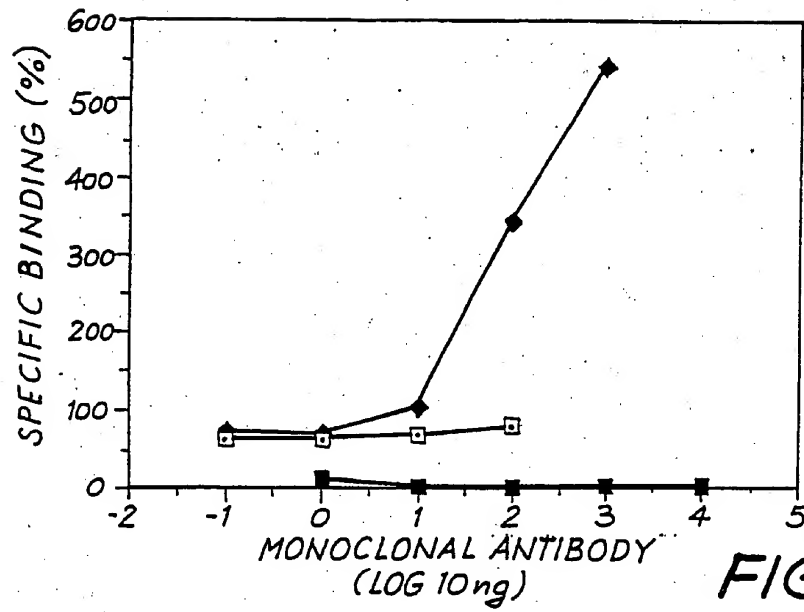
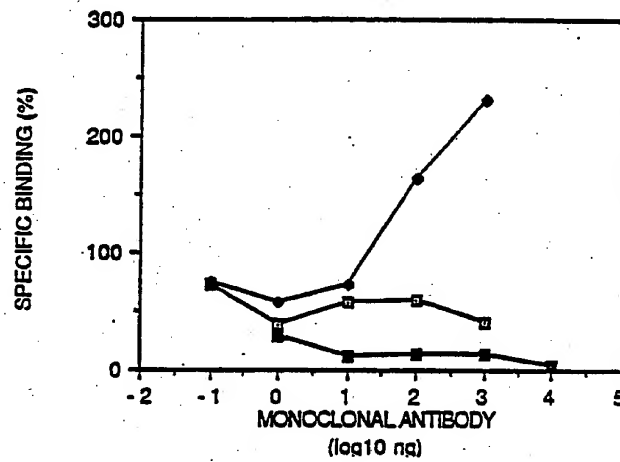


FIG.13

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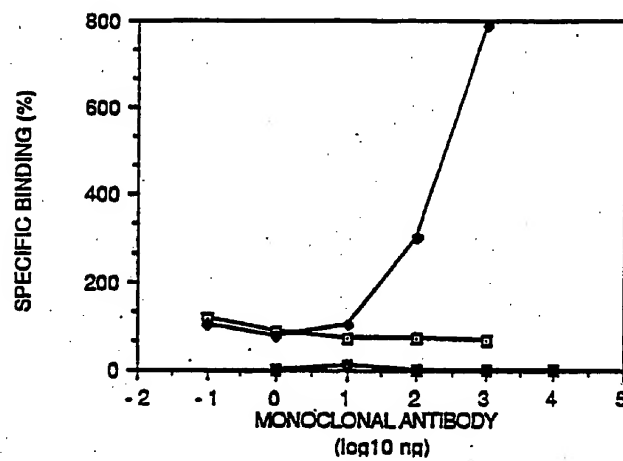
FIG. 14



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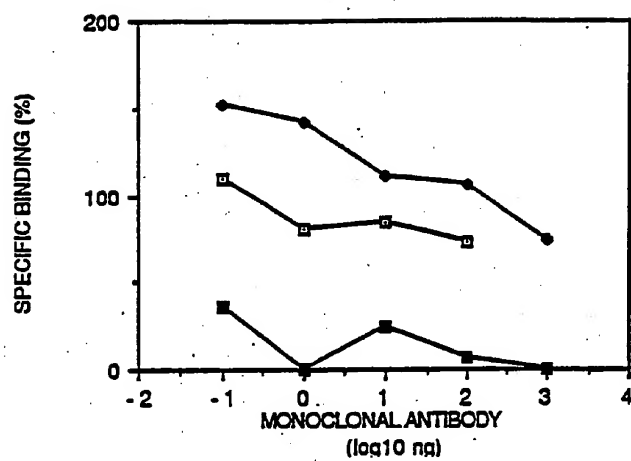
FIG. 15



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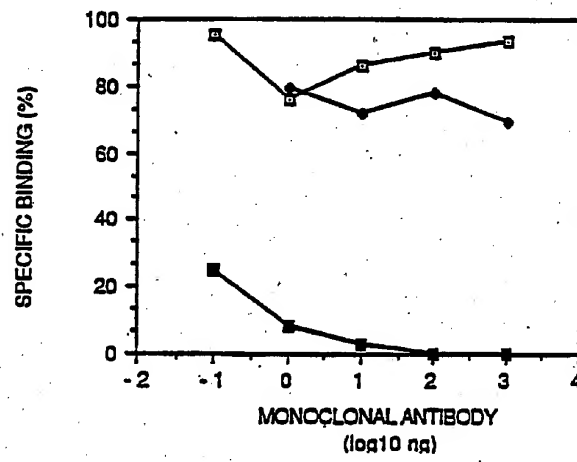
FIG. 16



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FIG. 17



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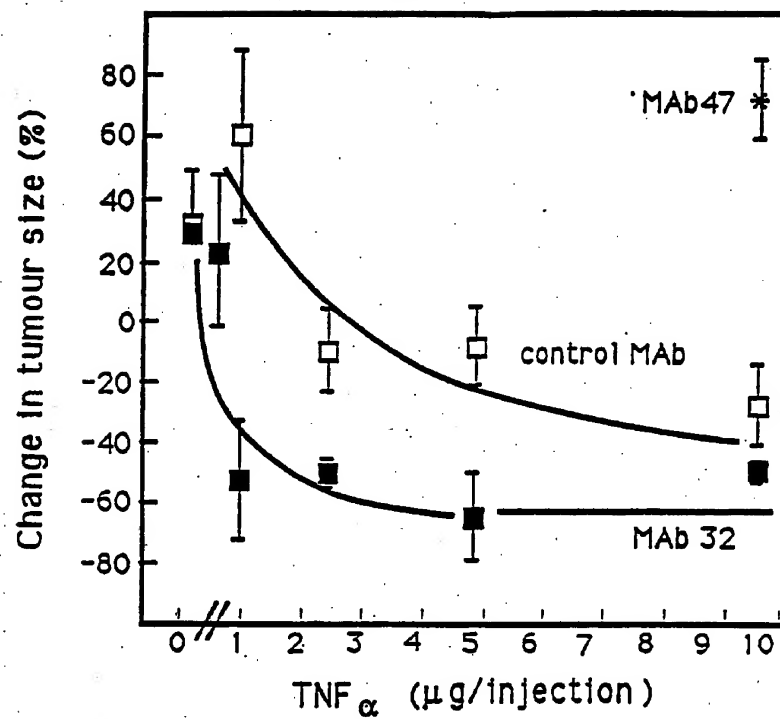
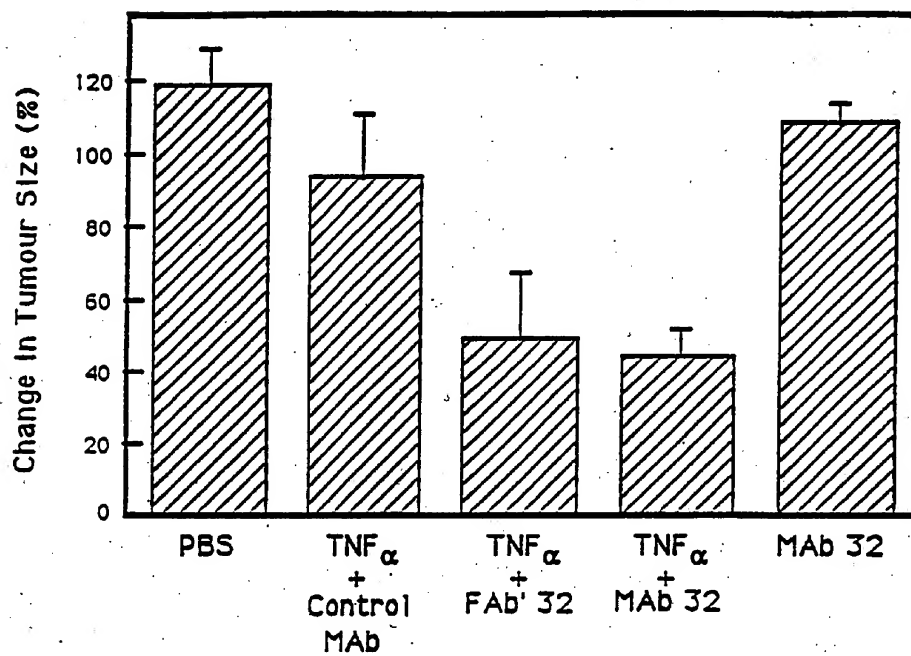


FIG. 18

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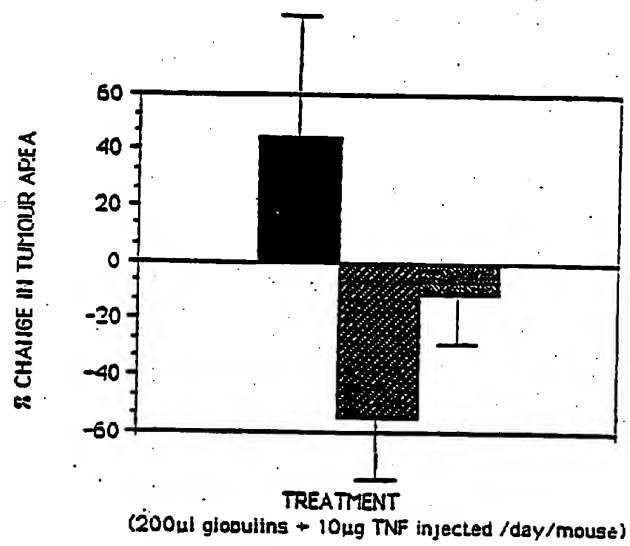
FIG. 19



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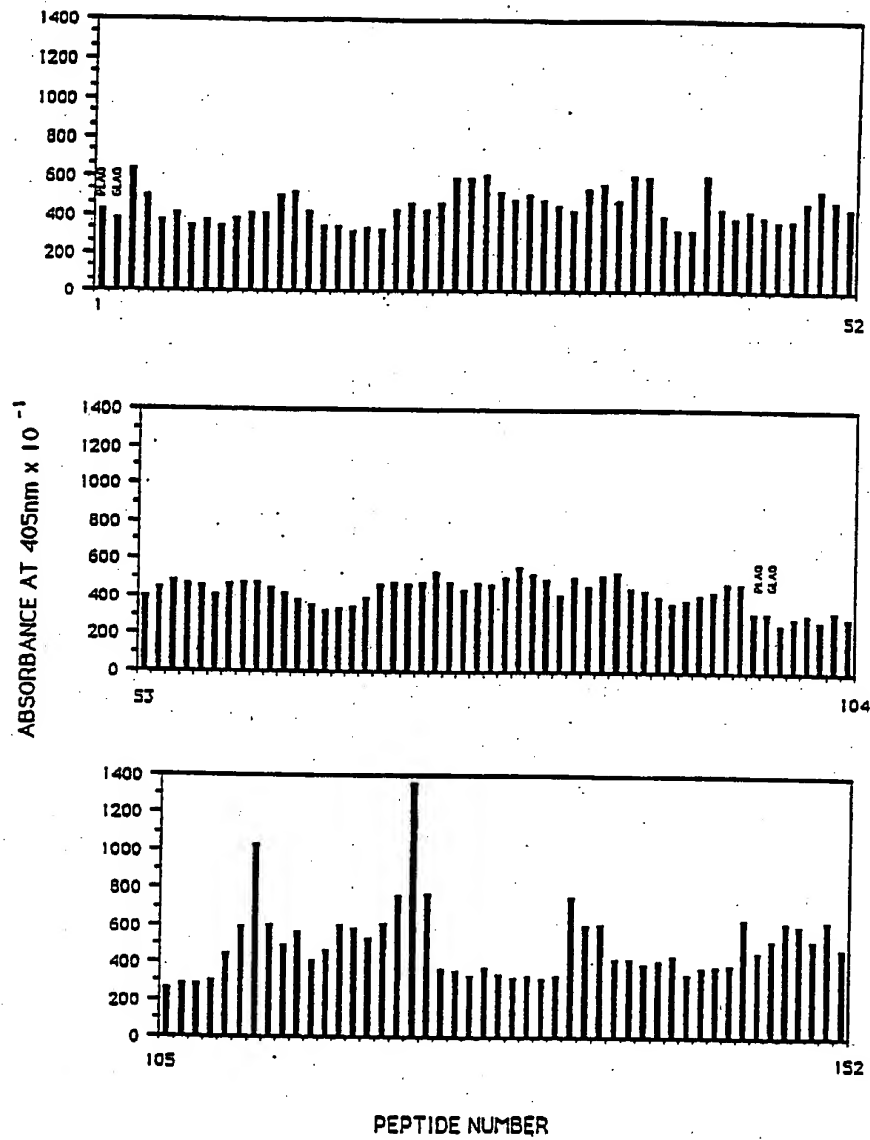
FIG. 20



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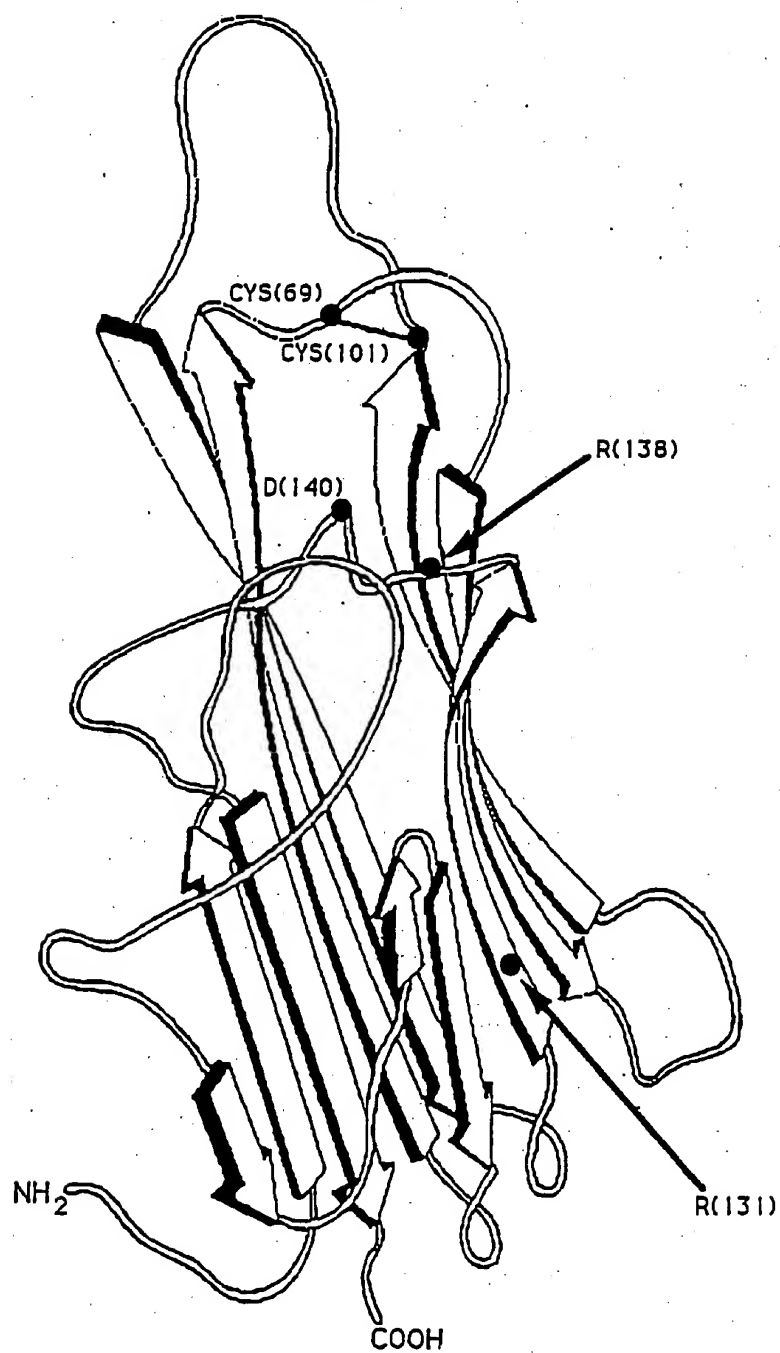
FIG. 21



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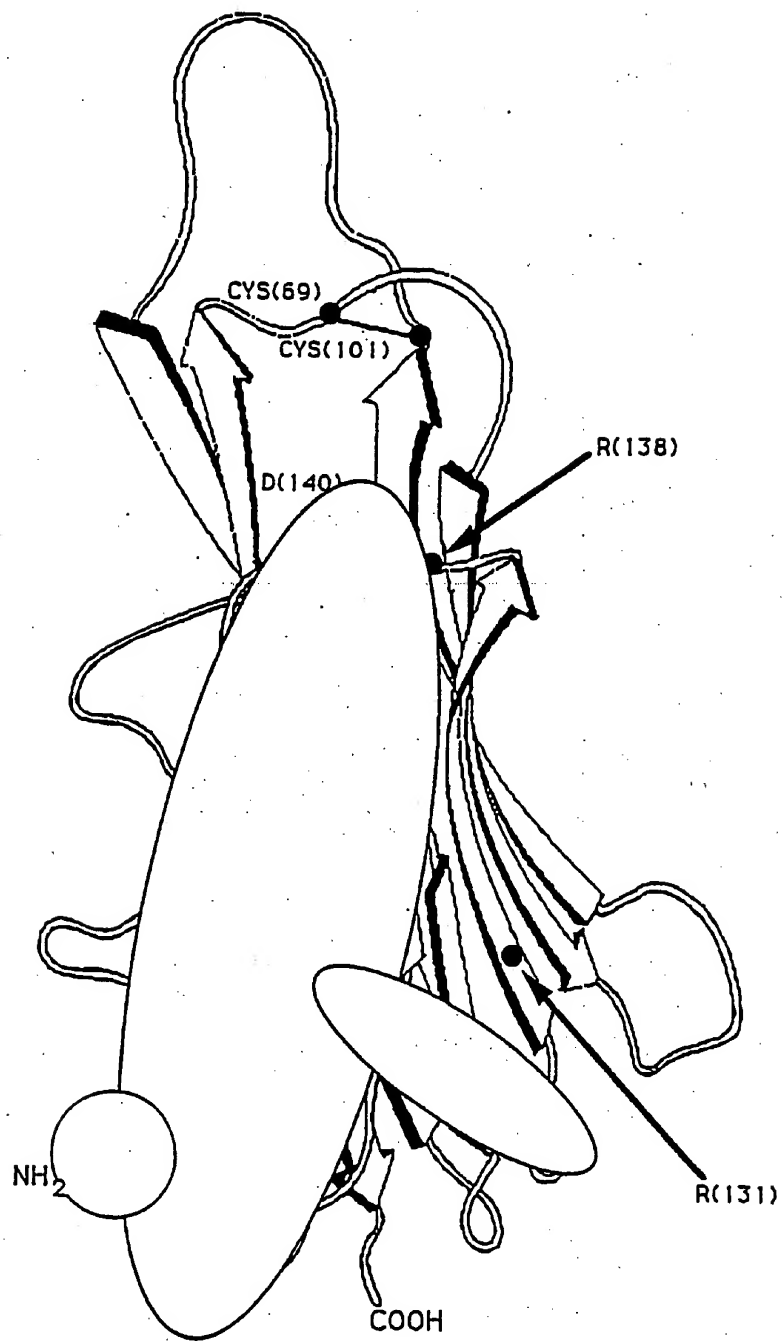
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FIG. 22

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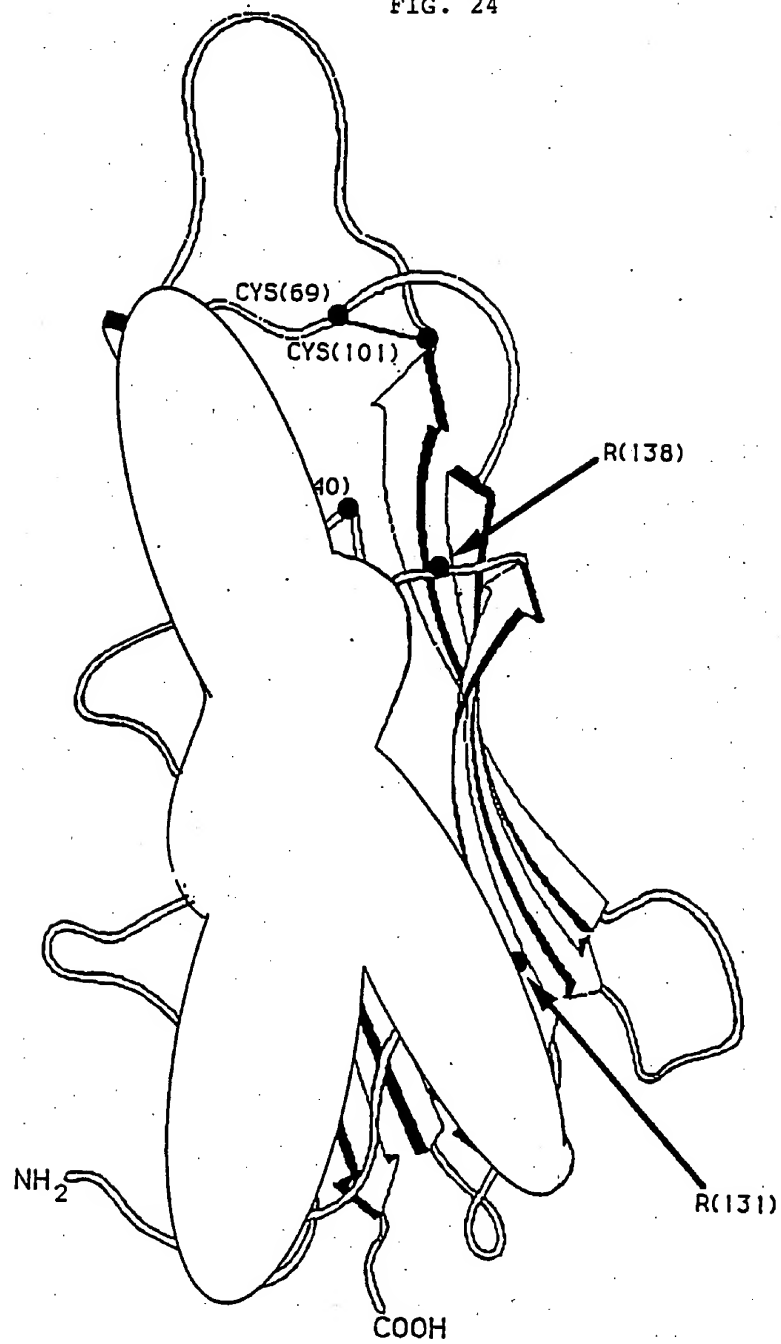
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FIG. 23

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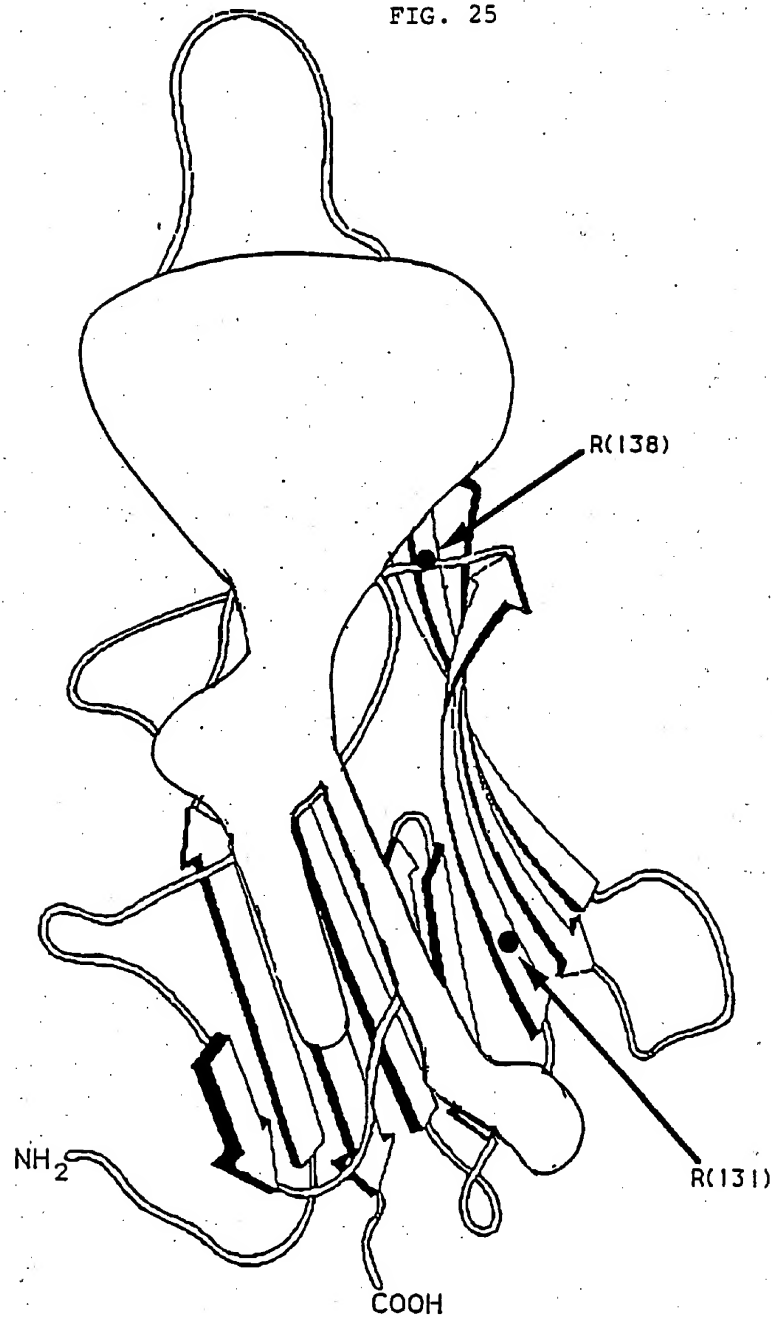
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FIG. 24

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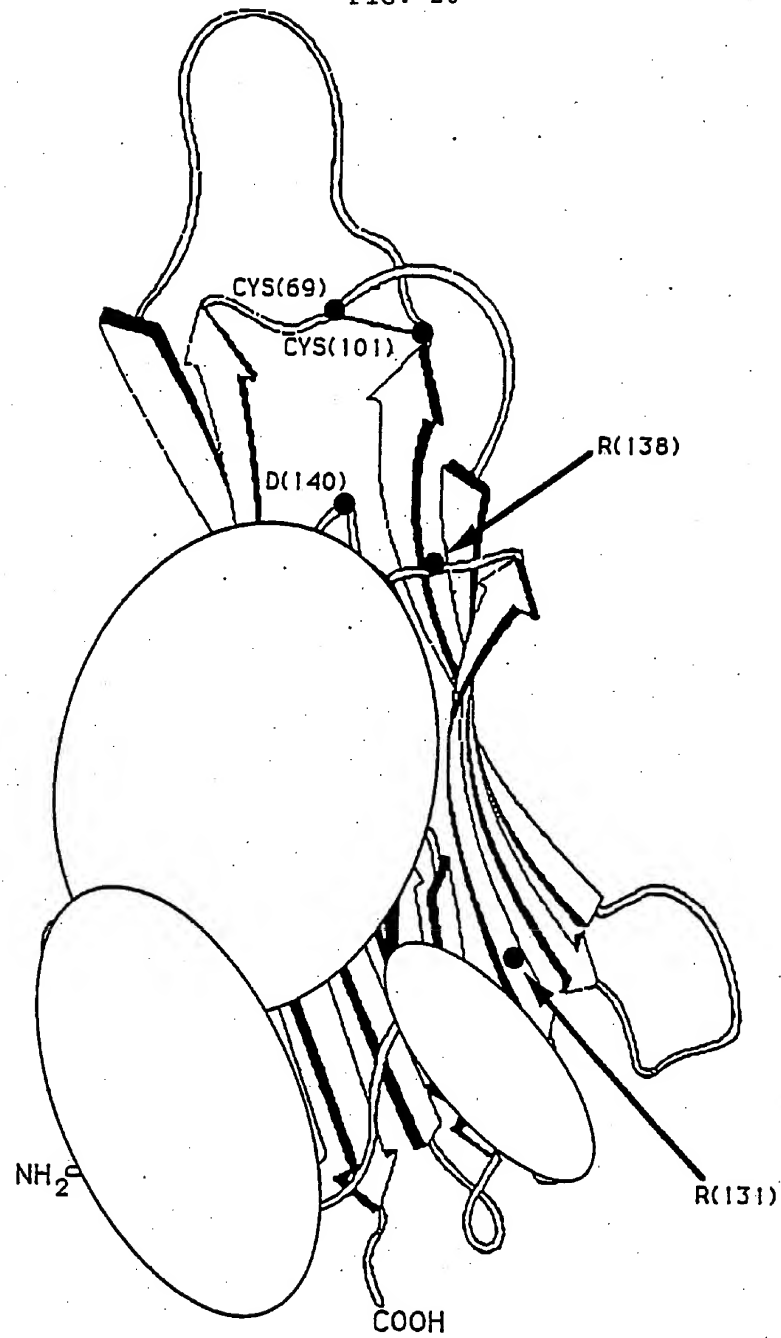
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FIG. 25

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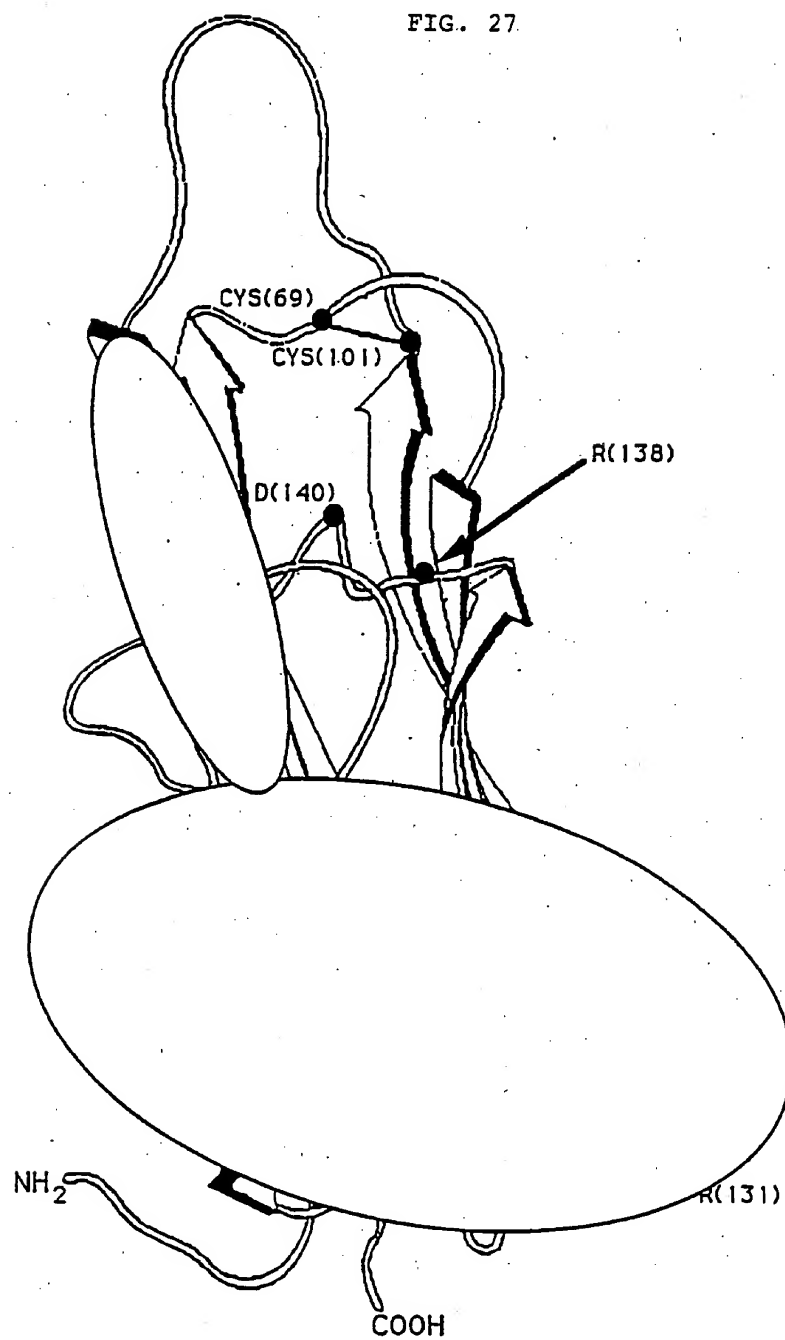
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FIG. 26

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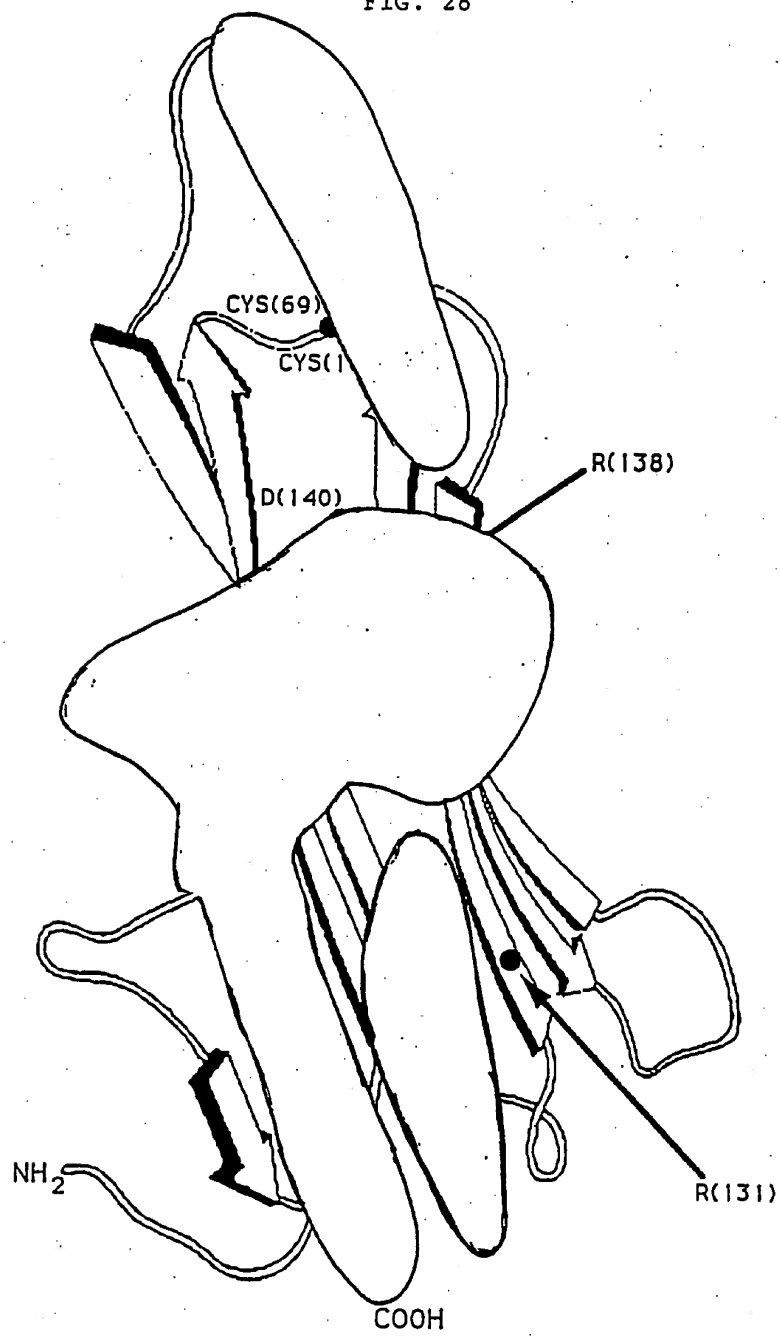
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FIG. 27.

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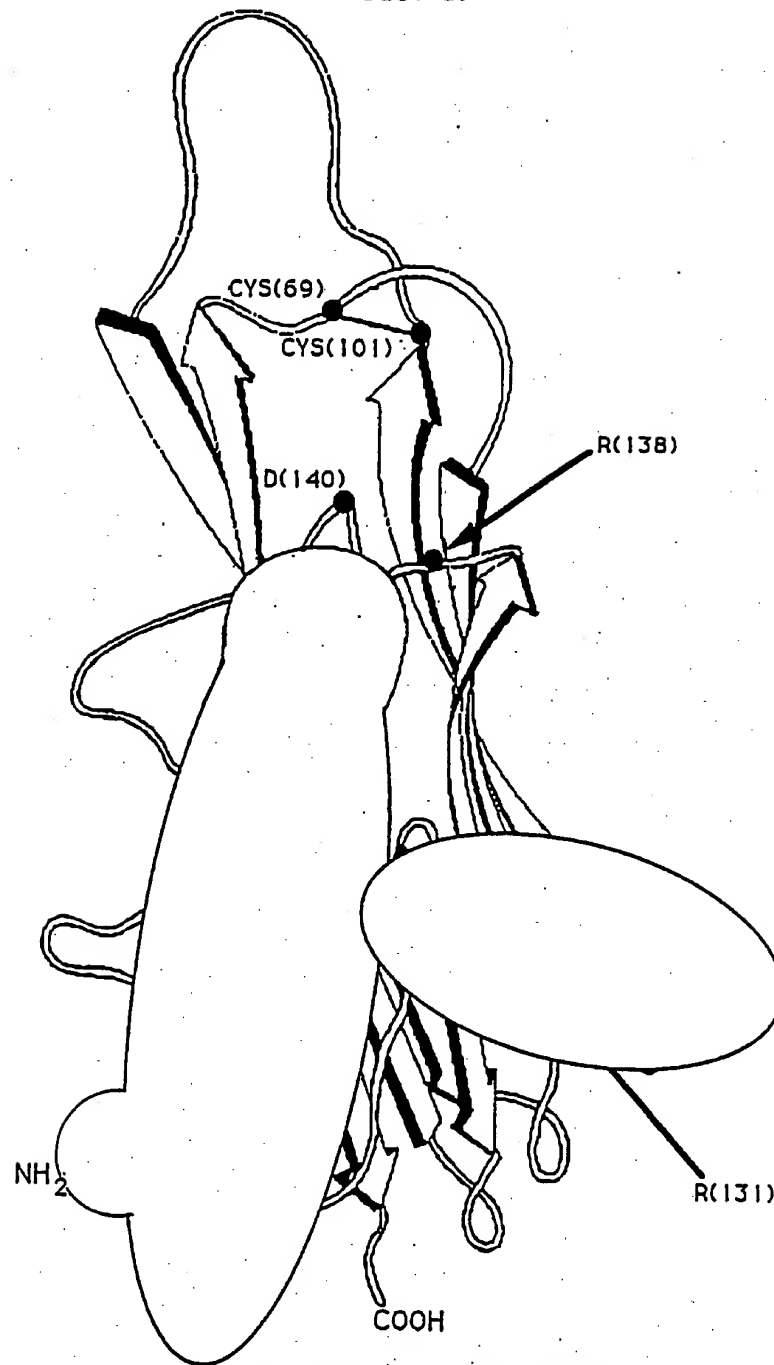
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FIG. 28

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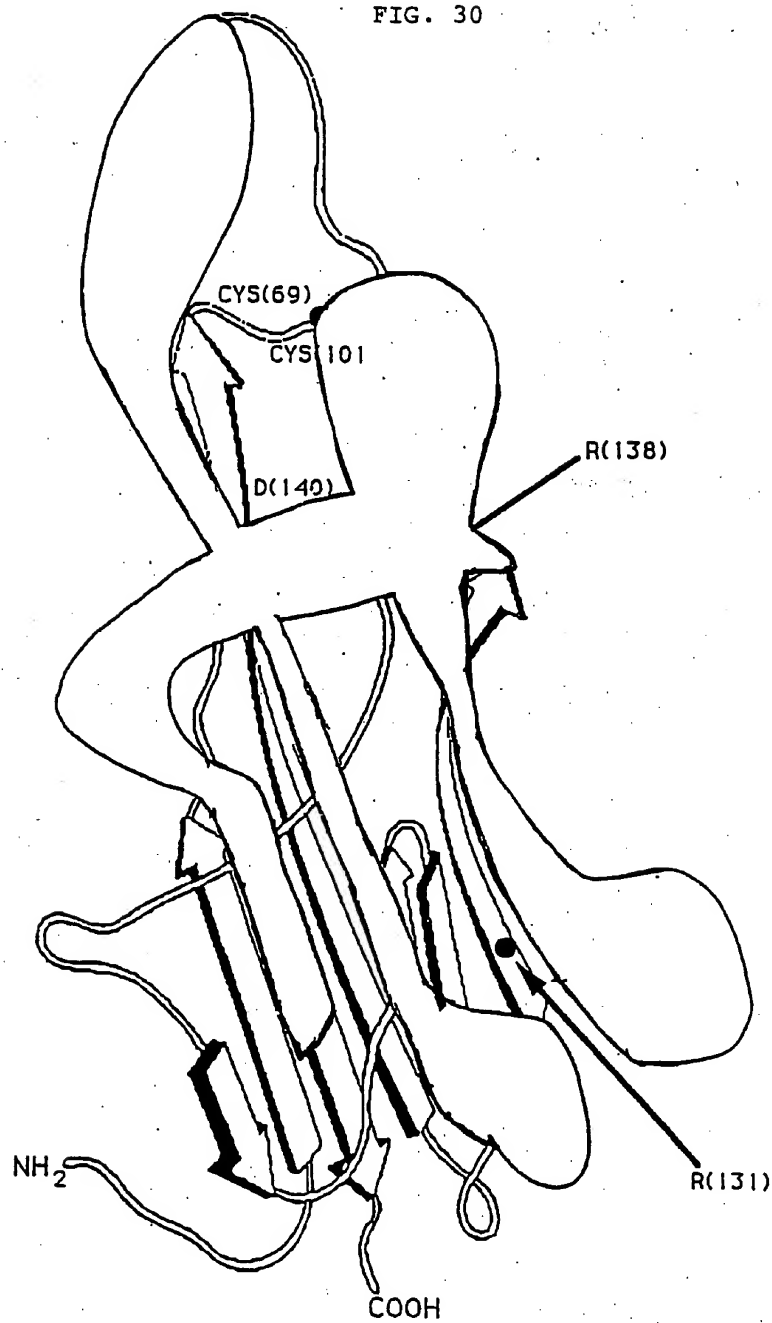
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FIG. 29

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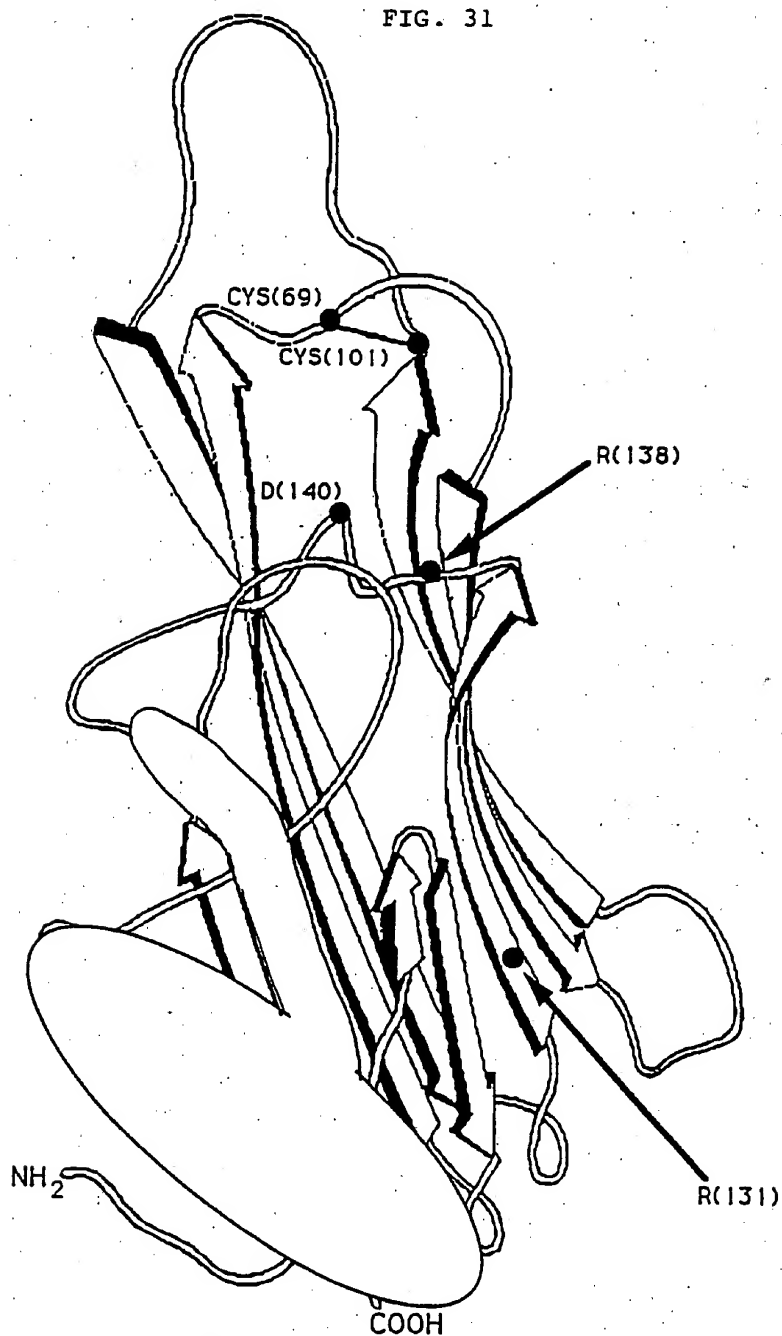
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FIG. 30

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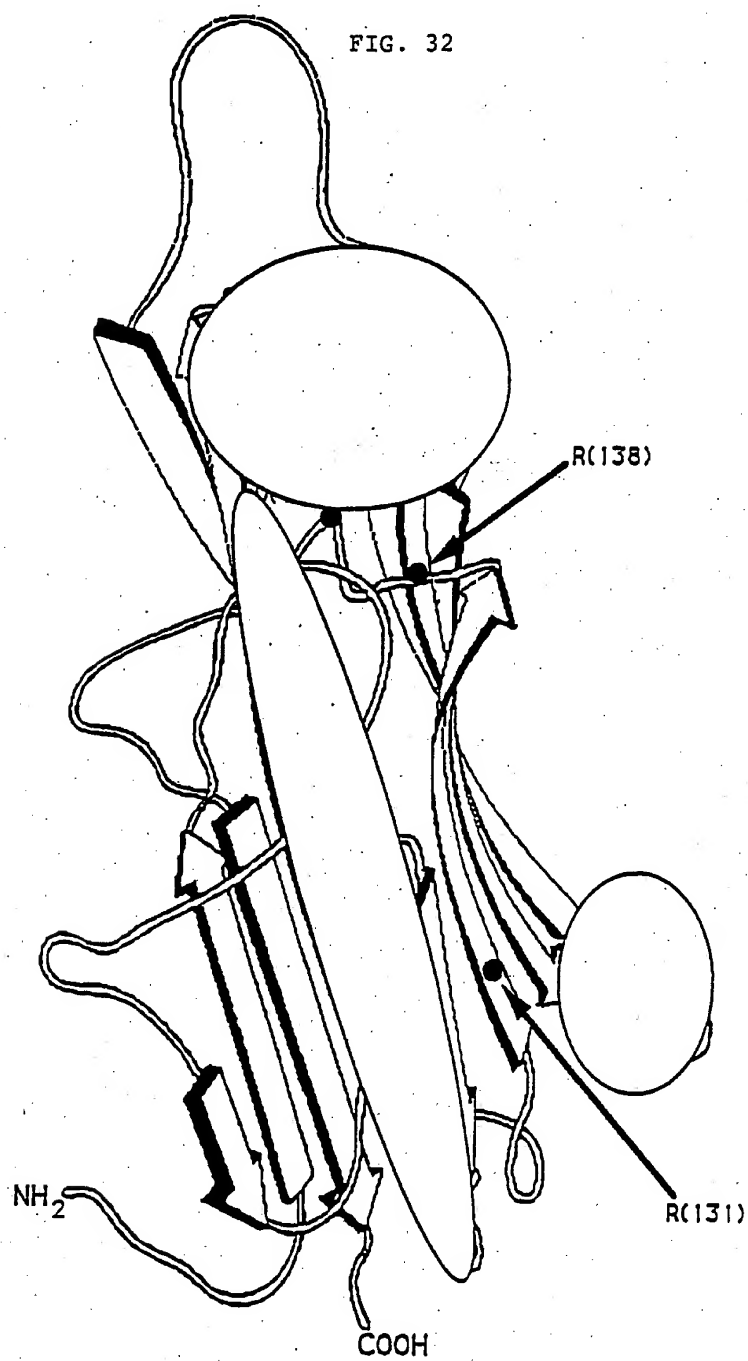
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FIG. 31

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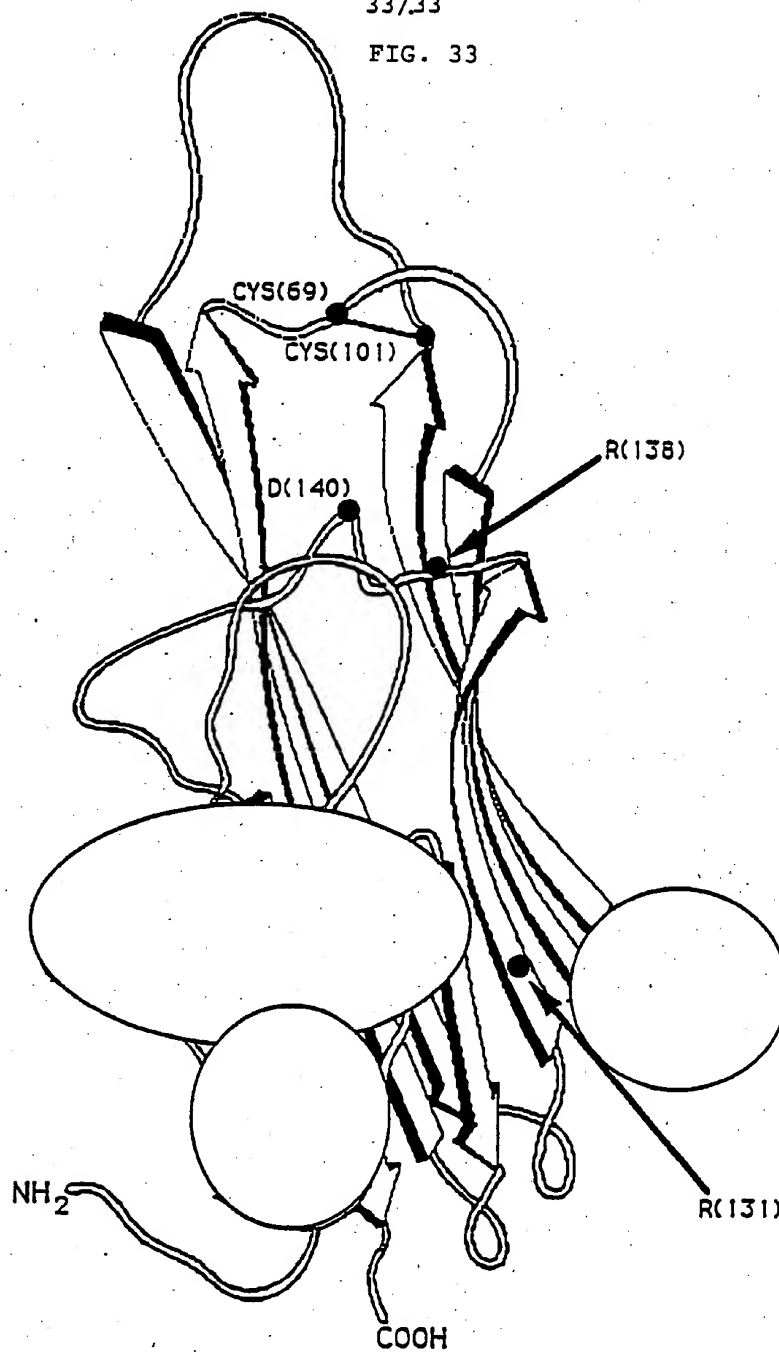
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FIG. 32

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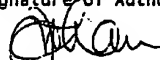
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FIG. 33

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 90/00337

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int. Cl. ⁵ C12P 21/08, C07K 15/28		
II. FIELDS SEARCHED		
Minimum Documentation Searched 7		
Classification System	Classification Symbols	
	WPI, WPIL USPA DERWENT DATABASE Keywords: HUMAN TUMOR NECROSIS FACTOR CHEMICAL ABSTRACTS Keywords: as above with ANTIBODY, RECEPTOR, LIGAND	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8		
AU : C12P 21/08		
III. DOCUMENTS CONSIDERED TO BE RELEVANT 9		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
P,X	EP,A1, 366043 (OTSUKA PHARMACEUTICAL CO., LTD) 2 May 1990 (02.05.90) see especially page 4	(1)
X	EP,A2, 288098 (TELJIN LTD) 26 October 1988 (26.10.88) see page 4	(27,67,68,70,71, 77,79)
Y	The Journal of Immunology, Vol.141, No.2, issued July 15,1988 (U.S.A.) Shalaby, M.R., et al, "The involvement of human tumor necrosis factors - α and - β in the mixed lymphocyte reaction" see page 499	(1)
Y	Hybridoma, Vol.6, No.4, issued 1987, (U.S.A.) , Fendly, B.M., "Murine monoclonal antibodies defining neutralizing epitopes on tumor necrosis factor", whole document	(1)
P,X	Patents Abstracts of Japan, C-678, page 112, JP,A, 1-268645 (TELJIN LTD) 26 October 1989 (26.10.89)	(1)
<p>* Special categories of cited documents: 10</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
9 November 1990 (09.11.90)	9 November 1990	
International Searching Authority	Signature of Authorized Officer	
Australian Patent Office	 J.H. CHAN	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 90/00337

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document
Cited in Search
Report

Patent Family Members

EP 366043 JP 2227095

EP 288088 JP 2001552

JP 1268645

END OF ANNEX